Severe Hand Foot and Mouth Disease in Vietnamese Children: Clinical Features and Management Strategies

Thesis

SEVERE HAND FOOT AND MOUTH DISEASE IN VIETNAMESE CHILDREN: CLINICAL FEATURES AND MANAGEMENT STRATEGIES

by

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Abstract

Hand, Foot and Mouth Disease (HFMD) plays a major role in childhood morbidity and mortality in Vietnam and the Asia-Pacific region. The spectrum of severe disease and predictors for poor outcome are not well understood, and the evidence-base for management of hypertension in severe cases is lacking.

In this thesis I describe the clinical features, their evolution over time, and the viral serotype/genotype findings, among 1272 HFMD cases admitted to PICU at the Hospital for Tropical Diseases (HTD), Ho Chi Minh City, during the the 2011-2012 Vietnam outbreak. Fever, skin rash, and myoclonic jerks happened in the early stage while the more severe features, including hypertension, tachypnea, irregular breathing, pulmonary edema and shock occurred after day 3. Most severe features were associated with EV-A71 infection rather than with Coxsackieviruses (CV). However having mouth ulcers without skin lesions was associated with CV infection. The C4 genogroup of EV-A71 was associated with more severe neurological involvement. Risk factors present within 24 hours of PICU admission that were associated with subsequent deterioration to severe outcome were skin lesions and tachypnea, while presence of mouth ulcers alone was protective.

I also describe the development and execution of a randomized controlled trial of a novel treatment, MgSO4, for severe HFMD with hypertension secondary to ANS dysregulation. However, only 26 of the planned 190 patients were enrolled before the outbreak was controlled, and with this small number we found no evidence of benefit in this trial. In another approach, I assessed the efficacy of MgOS4 on severe hypertension among 33 patients treated with open-label MgSO4 compared to 12 patients of similar clinical severity who did not receive this intervention, and found a significant reduction in mean arterial pressure among the MgSO4 recipients. However, It is clear that with the small sample size, these data are insufficient to address the important question originally posed. Using our pre-prepared protocol as a basis, a much larger trial could be developed at short notice in the event of another outbreak, to properly evaluate whether MgSO4 has a role in controlling the blood pressure in severe HFMD.

Co-Authorship

The work presented in this thesis was performed primarily by me, under the direction of my supervisors Dr Nguyen Van Vinh Chau and Professor Bridget Wills, during the course of my PhD project. Colleagues from the Hospital for Tropical Diseases (HTD), and the Emerging Infections (EI) Group, Dengue Group, Clinical Trials Unit (CTU) and Biostatistics Group at the Oxford University Clinical Research Unit (OUCRU) also contributed to this work as described below.

For the study of virological and clinical features associated with Hand, Foot and Mouth Disease (HFMD) and the assessment of predictors for severe outcome (Chapter 3), I developed the protocol, designed the case report form (CRF), and extracted the data from the hospital medical records together with a group of my HTD colleagues (Dr Huynh Trung Trieu, Dr Pham Thanh Giang, Dr Nguyen Thi Ngoc Bich, Dr Huynh Ngọc Thien Vuong, Dr Nguyen Thi My Tien, Dr Pham Thanh Quyen, Dr Nguyen My Chau, Dr Nguyen Thi Nghĩa) and the assistance of two study nurses (Ms Nguyen Minh Su and Ms Nguyen Truong An). Data entry was carried out by staff from the OUCRU Clinical Trials Unit, which I checked and verified all the queries later. The molecular diagnostics for enterovirus infection were largely performed by Nghiem My Ngoc (MSc) from HTD's Molecular Laboratory under the direction of Rogier Van Doorn (MD, PhD) and Le Van Tan (PhD) and his team from EI group. Some of the sequencing was performed by Dr David E. Wentworth from Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

For the randomized controlled trial (Chapter 4), I designed the protocol and CRF with Prof Wills, and developed all relevant Standard Operating Procedures with the assistance of Ms Laura Merson and Nguyen Duc Toan (MSc) from the Clinical Trials Unit (CTU). I supervised all aspects of the operation of the trial with assistance from Prof Wills, Dr Truong Huu Khanh, the co-PI and Head of Infectious Diseases Department from CH1, Mr Toan, former coordinator from CTU, and later, his replacement, Ms Le Thi Hoang Lan. All the clinical work was performed by myself and my study team, including experienced doctors and nurses from both hospital sites, with great assistance from the two study nurses, Ms Su and Ms An. The study drugs were prepared and allocated by Pharmacist Nguyen Bao Tran and her CTU colleagues. General safety monitoring was supervised by the CTU staff, while the

safety monitoring of plasma magnesium/calcium levels was carried out by a team of independent doctors, including Dr Nguyen Minh Nguyet (Dengue Group-OUCRU), Dr Nguyen Thi Hoang Mai (Malaria Deparment-HTD), Dr Nguyen Phu Huong Lan (Microbiology Department-HTD) and Dr Tran Thi Van Thinh (Malaria Group-OUCRU). Safety data were regular reviewed by the Independent Data and Safety Monitoring Board, including Professor Malcolm Molyneux (Liverpool School of Tropical Medicine, UK), Professor Nguyen Van Tuan (Garvan Medical Research Institute, Australia), Dr Simon Nadel (PICU, St Mary's Hospital and Imperial College London, UK), and Dr Nguyen Tran Nam (Infectious Disease Department, CH1, Vietnam. The viral diagnostics were performed by Dr Tan and his team (EI group-OUCRU), which I observed so as to learn about the procedures. The catecholamine ELISAs were performed by Huynh Thi Le Duyen (MSc; Dengue group-OUCRU). Neurodevelopment assessments were performed by Dr Pham Ngoc Thanh and her colleagues.

For the retrospective analysis of magnesium sulfate in management of severe HFMD (Chapter 5), I designed the CRF and extracted the data with help from Ms Su and Ms An.

For the statistical analysis and model development, I carried out all the main analyses in this thesis myself, but with advice from Dr. Marcel Wolbers (former Head of Biostatistics), Dr. Ronald Geskus (current Head of Biostatistics), and Prof Wills. I also had considerable support from Dr Phung Khanh Lam (Biostatistics Group), who guided me with both basic and advanced knowledge about R software. However, specific analyses for the correlations between catecholamine levels and cardiovascular parameters in Chapter 4, and development of the imputation model in Chapter 5, were done solely by Dr Lam.

I prepared the first draft of each chapter in this thesis, and am responsible for the structure and content, but I received considerable assistance from a number of OUCRU colleagues in the editing of these chapters for English.

Publications

A. Publications related to Hand Foot and Mouth Disease

1. **Qui PT**, Khanh TH, Trieu HT, Giang PT, Bich NN, Thoa le PK, Nhan le NT, Sabanathan S, Van Doorn R, Toan ND, Merson L, Dung NT, Khanh LP, Wolbers M, Hung NT, Chau NV, Wills B. *Intravenous magnesium sulfate for the management of severe hand, foot, and mouth disease with autonomic nervous system dysregulation in Vietnamese children: study protocol for a randomized controlled trial*. Trials. 2016 Feb 19;17:98.
2. Geoghegan JL, Tan le V, Kühnert D, Halpin RA, Lin X, Simenauer A, Akopov A, Das SR, Stockwell TB, Shrivastava S, Ngoc NM, Uyen le TT, Tuyen NT, Thanh TT, Hang VT, **Qui PT**, Hung NT, Khanh TH, Thinh le Q, Nhan le NT, Van HM, Viet do C, Tuan HM, Viet HL, Hien TT, Chau NV, Thwaites G, Grenfell BT, Stadler T, Wentworth DE, Holmes EC, Van Doorn HR. *Phylodynamics of Enterovirus A71-Associated Hand, Foot, and Mouth Disease in Viet Nam*. J Virol. 2015 Sep;89(17):8871-9.
3. Thanh TT, Anh NT, Tham NT, Van HM, Sabanathan S, **Qui PT**, Ngan TT, Van TT, Nguyet LA, Ny NT, Thanh le TM, Chai OK, Perera D, Viet do C, Khanh TH, Ha do Q,Tuan HM, Wong KT, Hung NT, Chau NV, Thwaites G, van Doorn HR, Van Tan L. *Validation and utilization of an internally controlled multiplex Real-time RT-PCR assay for simultaneous detection of enteroviruses and enterovirus A71 associated with hand foot and mouth disease*. Virol J. 2015 Jun 9;12:85.
4. Tan le V, Tuyen NT, Thanh TT, Ngan TT, Van HM, Sabanathan S, Van TT, Thanh le TM, Nguyet LA, Geoghegan JL, Ong KC, Perera D, Hang VT, Ny NT, Anh NT, Ha do Q, **Qui PT**, Viet do C, Tuan HM, Wong KT, Holmes EC, Chau NV, Thwaites G, van Doorn HR. *A generic assay for whole-genome amplification and deep sequencing of enterovirus A71*. J Virol Methods. 2015 Apr;215-216:30-6.
5. Sabanathan S, Tan L V, Thwaites L, Wills B, **Qui PT**, Rogier v D. H. *Enterovirus 71 related severe hand, foot and mouth disease outbreaks in South-East Asia: current situation and ongoing challenges*. J Epidemiol Community Health. 2014 Jun;68(6):500-2.

Poster abstract in international conference

Qui PT, Bridget Wills, Trieu T. Huynh, Giang T. Pham, Bich T. Nguyen Hung B. Nguyen Chau V. Nguyen. *The effect of Magnesium sulphate on autonomic dysregulation in enterovirus 71 related hand foot and mouth disease in Vietnamese children*. ASTMH annual meeting 2014. New Orleans, Lousiana, USA, astmh.org: p 138.

B. Publications related to other diseases

1. Trieu HT, Lubis IN, **Qui PT**, Yen LM, Wills B, Thwaites CL, Sabanathan S. *Neonatal Tetanus in Vietnam: Comprehensive Intensive Care Support Improves Mortality*. J Pediatric Infect Dis Soc. 2016 Jun;5(2):227-30. doi: 10.1093/jpids/piv059.
2. Lam PK, Trieu HT, Lubis IN, Loan HT, Thuy TT, Wills B, Parry CM, Day NP, **Qui PT**, Yen LM, Thwaites CL. *Prognosis of neonatal tetanus in the modern management era: an observational study in 107 Vietnamese infants*. Int J Infect Dis. 2015 Apr;33:7-11. doi: 10.1016/j.ijid.2014.12.011.
3. Lam PK, Tam DT, Diet TV, Tam CT, Tien NT, Kieu NT, Simmons C, Farrar J, Nga NT, **Qui PT,** Dung NM, Wolbers M, Wills B. *Clinical characteristics of Dengue shock syndrome in Vietnamese children: a 10-year prospective study in a single hospital.* Clin Infect Dis. 2013 Dec;57(11):1577-86. doi: 10.1093/cid/cit594.
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5. South East Asia Infectious Disease Clinical Research Network. *Effect of double dose oseltamivir on clinical and virological outcomes in children and adults admitted to hospital with severe influenza: double blind randomized controlled trial*. BMJ. 2013 May 30;346:f3039. doi: 10.1136/bmj.f3039.

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Finally, I would like to say thanks to my loving family for being by my side all the time.

Abbreviations

ng	Nanogram
pg	Picogram
µg	Microgram
µM	Micromole
µL	Microliter
AFP	Acute Flaccid Paralysis
ANS	Autonomic Nervous System
ASTMH	The American Society Of Tropical Medicine And Hygiene
AUC	Area Under The Curve
BE	Brainstem Encephalitis
BHK-21	Baby Hamster Kidney 21
BP	Blood Pressure
CCL27	Chemokine (C-C Motif) Ligand 27
CD 4+	Cluster Of Differentiation 4
CD14+	Cluster Of Differentiation 14
CDC	The Centers For Disease Control And Prevention
CH1	Children's Hospital 1
CI	Confidence Interval
CK-MB	Creatine Kinase-MB
CLIRES	Clinical Research Data Management System
CNS	Central Nervous System
Cp	Crossing Point
CPE	Cytopathic Effect
CRF	Case Record Form
CRP	C Reactive Protein
CRT	Capillary refill time
CT scan	Computerized Tomography Scan
CV	Coxsackievirus
CV-A	Coxsackievirus A

CV-B	Coxsackievirus B
CVC	Central Venous Catheter
CVVH	Continous Veno-Venous Hemofiltration
CTCAE	Common Terminology Criteria for Adverse Events
CXCL-1	The Chemokine (C-X-C Motif) Ligand 1
DBP	Diastolic Blood Pressures
DNA	Deoxyribonucleic Acid
DOI	Day Of Illness
DSMB	Data and Safety Monitoring Board
EAV	Equine Arteritis Virus
ECG	Electrocardiogram
EEG	Electroencephalogram
ELISA	Enzyme-Linked Immunosorbent Assay
EV	Enterovirus
EV-A71	Enterovirus A71
FBC	Full Blood Count
FDA	The Food And Drug Administration
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HA	Herpangina
HDU	High Dependency Unit
HEV	Human Enterovirus
HFMD	Hand Foot and Mouth Disease
HR	Heart Rate
HRV	Heart Rate Variability
HTD	Hospital For Tropical Diseases
HTN	Hypertension
IC	Internal Control
ICAM-1	Intercellular Adhesion Molecule 1
ICH-GCP	The International Conference On Harmonisation - Guideline For Good Clinical Practice

IBP	Invasive Blood Pressure
ID	Identification
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IL	Interleukin
INFγ	Interferon-gamma (γ)
IQR	Interquartile Range
IP-10	IFN-Gamma-Inducible Protein 10
IV	Intravenous
IVIG	Intravenous Immunoglobulin
LLC-MK	Lipid Metabolism Of Monkey Kidney
LYM	Lymphocyte
MAP	Mean Arterial Pressure
MCP-1	Monocyte Chemoattractant Protein-1
MPP 1b	Macrophage Inflammatory Protein 1-Beta
MgSO4	Magnesium Sulfate
MoH	Ministry Of Health
MRI	Magnetic Resonance Imaging
NCI	The National Cancer Institute
NA	Not available
NEU	Neutrophil
NLRs	NOD-Like Receptors
OR	Odds Ratio
OUCRU	Oxford University Clinical Research Unit
PBMCs	Peripheral Blood Mononuclear Cell
PICU	Pediatric Intensive Care Unit
PIS	Patient Information Sheet
PLT	Platelet
RCT	Randomized Controlled Trial
PKR	Protein Kinase Regulated By RNA

RD	Rhabdomyosarcoma
RLRs	Retinoic Acid-Inducible Gene (Rig-I)-Like Receptors
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SBP	Systolic Blood Pressure
SAE	Serious adverse event
SOP	Standard Operating Procedure
T1IFNs	Type I Interferons
TLRs	Toll-Like Receptors
TNF	Tumor Necrosis Factor
VP	Viral Protein
Vpg	Virion Protein
UTR	Untranslated Region
UI	International Unit
US	United State Of America
WHO	World Health Organization

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Chapter 1

INTRODUCTION

1.1 Human enterovirus infections

Human enterovirus infections affect many millions of people worldwide each year. Although they are named for their transmission route through the intestine, enteroviral infections have a broad range of presentations involving many organs and systems (e.g. fever with rash, upper respiratory infections, encephalitis, pericarditis), and gastrointestinal symptoms are rarely prominent. Many infections are very mild and may go unnoticed, but given the variety of possible signs and symptoms they are also on the differential diagnosis list of a wide range of illnesses, some of which can be very severe. One particularly important virus, poliovirus, was extensively researched in the twentieth century largely due to the severity of illness it caused. As a result, effective vaccines that were developed have been deployed globally with massive worldwide effort, and polio has now been virtually eradicated from both developed and developing countries. However, non-polio enteroviruses have received much less attention, and remain major causes of disease affecting a large number of people, especially children, around the world. Since this thesis relates to hand, foot and mouth disease (HFMD), the main focus of the introduction will be on non-polio enteroviruses.

1.1.1 Virology, physical characteristics and classification

Enteroviruses belong to the family *Picomaviridae* and the genus *Enterovirus*. The factor that distinguishes enteroviruses from other picornaviruses (i.e. rhinoviruses) is their stability in low pH environments. Enteroviruses persist in ether and alcohol solutions and can cause infection in pH 3-10 environment, but are inactivated at a temperature above 50^0 C [1]. All enteroviruses share these common physical and biological characteristics. The non-enveloped virion diameter is about 30 nm, and the icosahedral viral capsids are composed of four structural proteins namely viral protein (VP) 1, VP2, VP3 and VP4. Each capsid contains a total of about 60 structural proteins.

The virus genome is a single stranded positive-sense RNA molecule of 7,5 kb in length.

This genome encodes for the structural proteins (VP1 - VP4), and nonstructural proteins (including RNA polymerase and protease). The protein coding region is flanked by the 5' untranslated region (UTR) and the 3'UTR. The former is a highly conserved region across enterovirus genomes (Figure1.1). This genome starts with 5'UTR that contain Internal Ribosome Entry Site (IRES), the protein coding region, linked to Virion protein (Vpg) and ends with 3'UTR that contains a pseudoknot and the poly (A) tail. These two play a role in starting and ending the translation and replication of the virus [1].

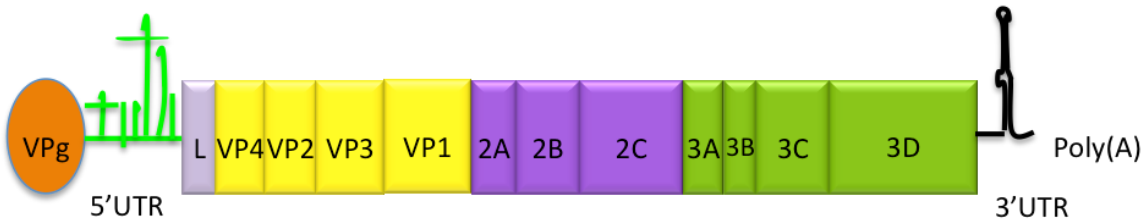


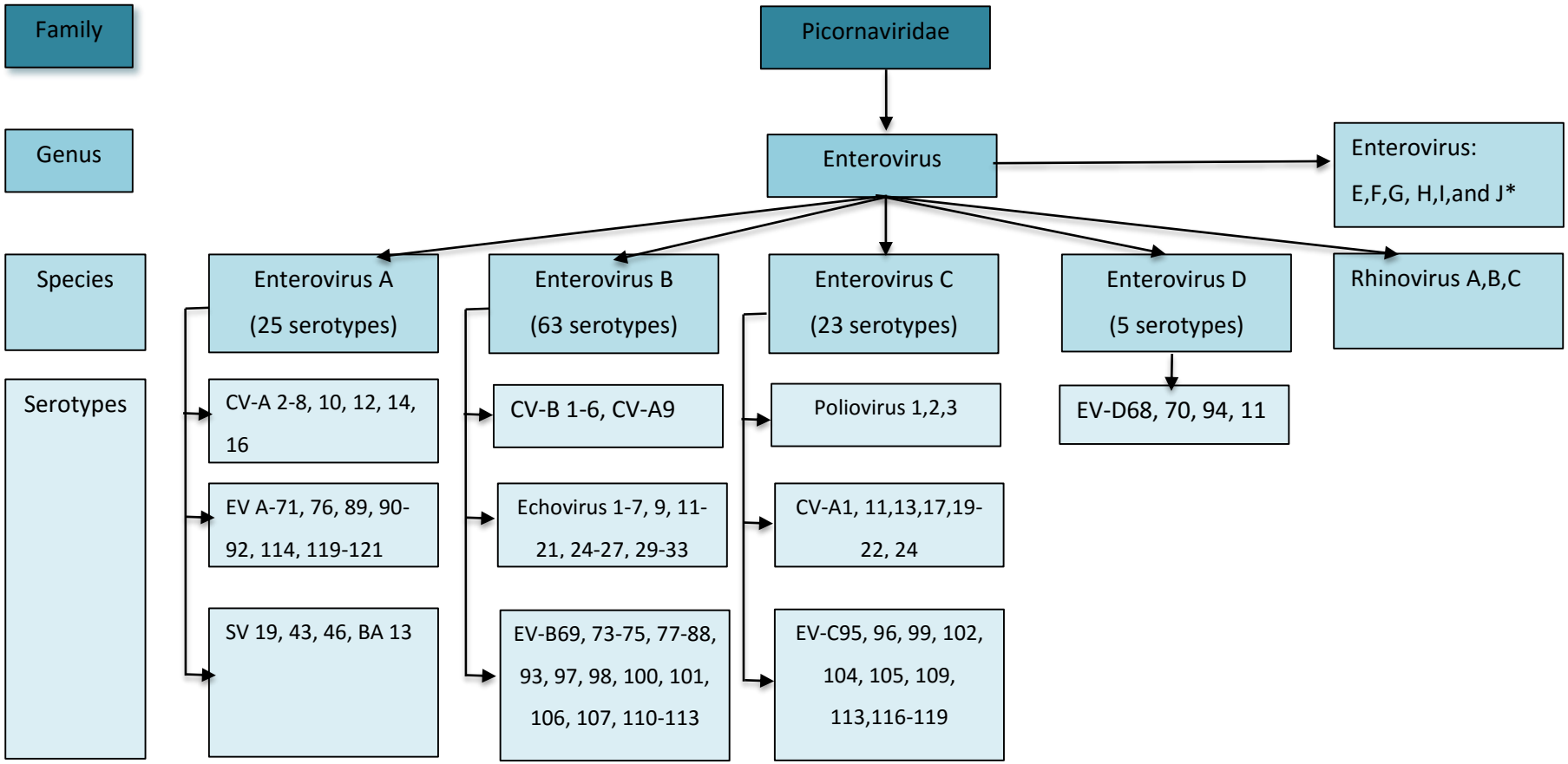
Figure 1.1: The picornavirus genome (reproduced from [1])

VPg: Virion protein, UTR= Untranslated region, L= leading protein code; VP1-VP4 encode for viral capsid proteins; 2A, 3C and 3D encode for proteins functioning as proteins processing; 2B, 2C, 3A, 3B, 3C, and 3D encode for proteins involving genome replication of enteroviruses.

The enterovirus group originally included 72 serotypes that were identified by their neutralization reactions with specific antibodies, and these were classified into 5 subgroups based on different susceptibility of the host and pathogen [2, 3]. Recently, a number of new serotypes have been identified by molecular biological methods, and the non-poliovirus enteroviruses have been reclassified into 13 species that are divided into 4 main human virus groups, now named EV-A, EV-B, EV-C, and EV-D, plus 6 animal enterovirus groups, ie. EV-E, F, G, H, I and J, and 3 rhinovirus groups (A, B, and C) based on the homogenous characteristics of VP1 protein encrypted RNA [4-6]. However, among all these, around 10 to 15 serotypes are responsible for the majority of human diseases, as described in Table 1.1, section 1.1.5 [7, 8].

Figure 1.2: Enterovirus classification

(derived from http://www.picornaviridae.com/enterovirus/ev-a/ev-a.htm)



*: Enterovirus species E, F (former name *bovine enterovirus*), Enterovirus species G (former name *porcine enterovirus*), H, J (former name *simian enterovirus A) and I*, are commonly seen in animals

EV=enterovirus, CV= Coxsackievirus; SV= Simian enterovirus; BA= baboon enterovirus

1.1.2 General epidemiology of human enterovirus infections

Enteroviruses are ubiquitous globally and circulate around the world, but different serotypes may be endemic in different regions. For example, while EV-A71 has caused large outbreaks of HFMD in the Asia-Pacific region, EV-D68 has emerged in recent years to cause prominent outbreaks of respiratory infections in the US and Europe [9, 10], and Eastern Asia [11, 12]. Young children under 5 years old are usually more susceptible to enterovirus infection than adults. Many studies have revealed that enterovirus infections happen more frequently in males than in females. The male-to-female ratio is between 1.2 and 2.5: 1 [13].

Transmission

These viruses are transmitted from person to person when virus is shed from the gastrointestinal or respiratory tracts of an infected individual. Typically, transmission occurs by the faecal-oral route, but enteroviruses are also found in body secretions; for example EV70 was demonstrated to be present in tears, contributing to an outbreak of conjunctivitis in Samoa [14], while EV68 was found in respiratory secretions resulting in spread between patients in Cambodia and Philippines [15, 16].

Transmission between humans occurs both directly and indirectly. Direct transmission occurs when people come into immediate contact with infected material, such as faeces during diaper changing. Indirect transmission occurs when individuals, particularly children, come into contact with contaminated water, food, toys, that they put in their mouths. Poor hygiene, particularly failure to wash hands before meals contributes to this transmission cycle [12, 17-20]. Longitudinal surveys indicate that secondary infections occur in 53% of exposed household contacts, and confirm that Infants, especially those in diapers, were the main source of infection. The role of virus persistence in both symptomatic and asymptomatic individuals who are basically healthy may be important but remains to be clarified [21]. However it is clear that chronic viral persistence can occur in immunocompromised individuals, thus increasing the risk for secondary virus transmission [22-24].

Relationships between serotypes, seasonality, and outbreaks

Enteroviruses are distributed worldwide, with transmission patterns influenced by both season and climate. Monitoring circulating enteroviruses can be helpful to identify changes in the predominant serotypes, which may be accompanied by large-scale outbreaks. For example, epidemiological surveillance reports from 2002 to 2004 in the United States were used to examine temporal and geographic patterns of serotypes; the two predominant enteroviruses, echoviruses 9 and 30, which had not been common previously, accounted for more than half of all enterovirus detections during this time and the increased surveillance reports coincided with peaks in hospitalizations for aseptic meningitis [25].

Reported case numbers can increase dramatically in comparison to prior/baseline incidence data during an outbreak [26]. Global epidemics also occur: e.g. associated with echovirus 10 in the end of 1950s; associated with echovirus 11 in 1979-1980; and an EV 70 conjunctivitis outbreak commencing in 1969. Enterovirus infections occur throughout the year but numbers usually increase in the warmer seasons. Published surveillance data show that outbreaks often happen during the summer in tropical countries [27], and in summer and fall in northern countries [28, 29]. Humidity is also thought to play a role in outbreak occurrence [27, 29, 30], and there are concerns that the global warming phenomenon may result in increased incidence and a wider geographical distribution of enterovirus infections [30, 31]. A seasonal model developed by a Japanese group almost exactly matched annual fluctuations in local case numbers and predicted that warmer climate conditions would lead to an increased number of herpangina (HA) and HFMD cases, simulating the impact of global warming [32].

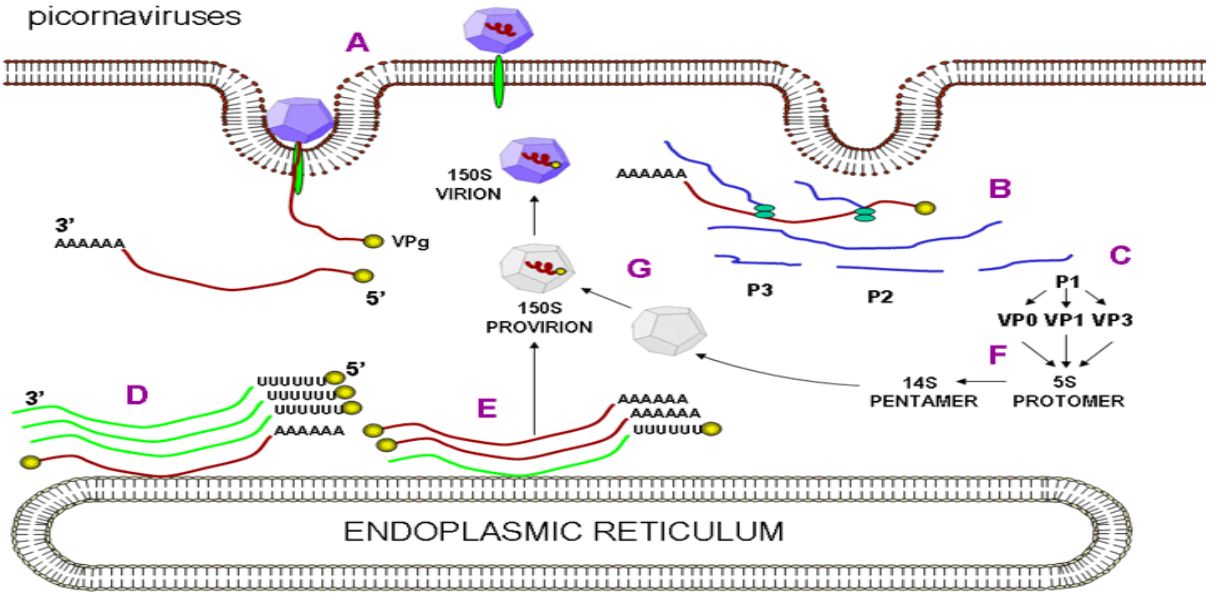
1.1.3 Pathogenesis

Cellular invasion, replication and dissemination

After infection via the oral route, enteroviruses replicate initially in the submucosal tissues of the distal pharynx and the gut, particularly the terminal ileum. Recently, they were also found replicating in tonsillar crypt squamous epithelium [33]. Although

incubation times vary depending on the virus involved and the particular clinical and syndrome, average times are around 3-5 days, after which fever develops in those who become symptomatic. During this period the virus migrates to regional lymph nodes and begins replicating there. Viral particles may therefore be shed before the onset of symptoms.

Different enteroviruses attach to cell membranes via particular receptors, and these receptors are both virus and host specific, eg. P-selectin glycoprotein ligand-1 (PSGL-1), Scavenger receptor class B (SCARB2) are receptors for EV-A71 while Decay-accelerating factor (CD55) is a receptor for certain Coxsackieviruses (CV), echoviruses, and enterovirus 70, therefore determining host susceptibility [1]. After attachment, a conformational change takes place in the viral capsid proteins, resulting in formation of a pore in the cell membrane through which viral RNA is injected into the cell. The RNA un-coats rapidly and the whole process of replication is complete on average within 8 hours, when the cell dies and lyses to release the new viral particles (Figure 1.3).



A= Adsorption and penetration, B= translation and synthesis of viral proteins; C= Cleavage of the precursor protein; F= formation of viral capsid; D= negative-sense RNA replication, E= positive-sense RNA replication, G: Assembly; P: precursor protein, VP: viral protein

Figure 1.3: The invasion and replication of enterovirus species

(http://www.microbiologybook.org/virol/picorn-rep.gif)

After about 5 to 10 hours whole virions may be seen on electron microscopy; each infected cell contains around 10^4-10^5 virions, and between 0.1-10% of them are infectious [34, 35].

Knowledge about replication and dissemination of enteroviruses is based on early research in animal models conducted by Bodian, Sabin and others (Figure 1.4) [36, 37]. During the initial replication phase involving the pharynx and terminal ileum, relatively low level primary viremia results, following which viral spread occurs to lymph nodes and organs of the reticulo-endothelial system. Viral replication at these sites causes a secondary viremia that coincides with the onset of symptoms and with viral dissemination to other target organs, potentially including the central nervous system (CNS) that will be discussed in the next section. Infectious virus is shed from the upper respiratory tract for about 1-3 weeks and is eliminated in stool for 3-11 weeks [38]. The most infectious period for the community is around 2 weeks after infection [39].

Invasion into the central nervous system (CNS)

First of all, enteroviruses invade and replicate in the gastrointestinal tract, after which they disseminate to their target organs/tissues via specific pathways. The broad spectrum of neurological manifestation of enterovirus infection, including aseptic meningitis, brainstem encephalitis (BE) and polio-like myelitis suggest that non-polio entervirus, including EV-A71, can invade the CNS either through a disintegrated blood–brain barrier or via retrograde axonal spread along cranial or peripheral nerves. Most previous studies of the way that enteroviruses invade the CNS were focused on the poliovirus that could invade into the CNS through neuromuscular junctions [40, 41]. However, it has demonstrated that EV-A71 enters the CNS via retrograde axonal transport pathways in mice [42]. Another possible pathway is through intermediary vehicles such as CV infected Mac-3+ mononuclear cells, or EV-A71 infected CD14+ cells, dendritic cells, and PBMCs; these cells have the ability to invade the CNS through the choroid plexus epithelium [43-45]. However, evidence to support viral entry to the CNS utilizing infected immune cells CNS remains limited until now.

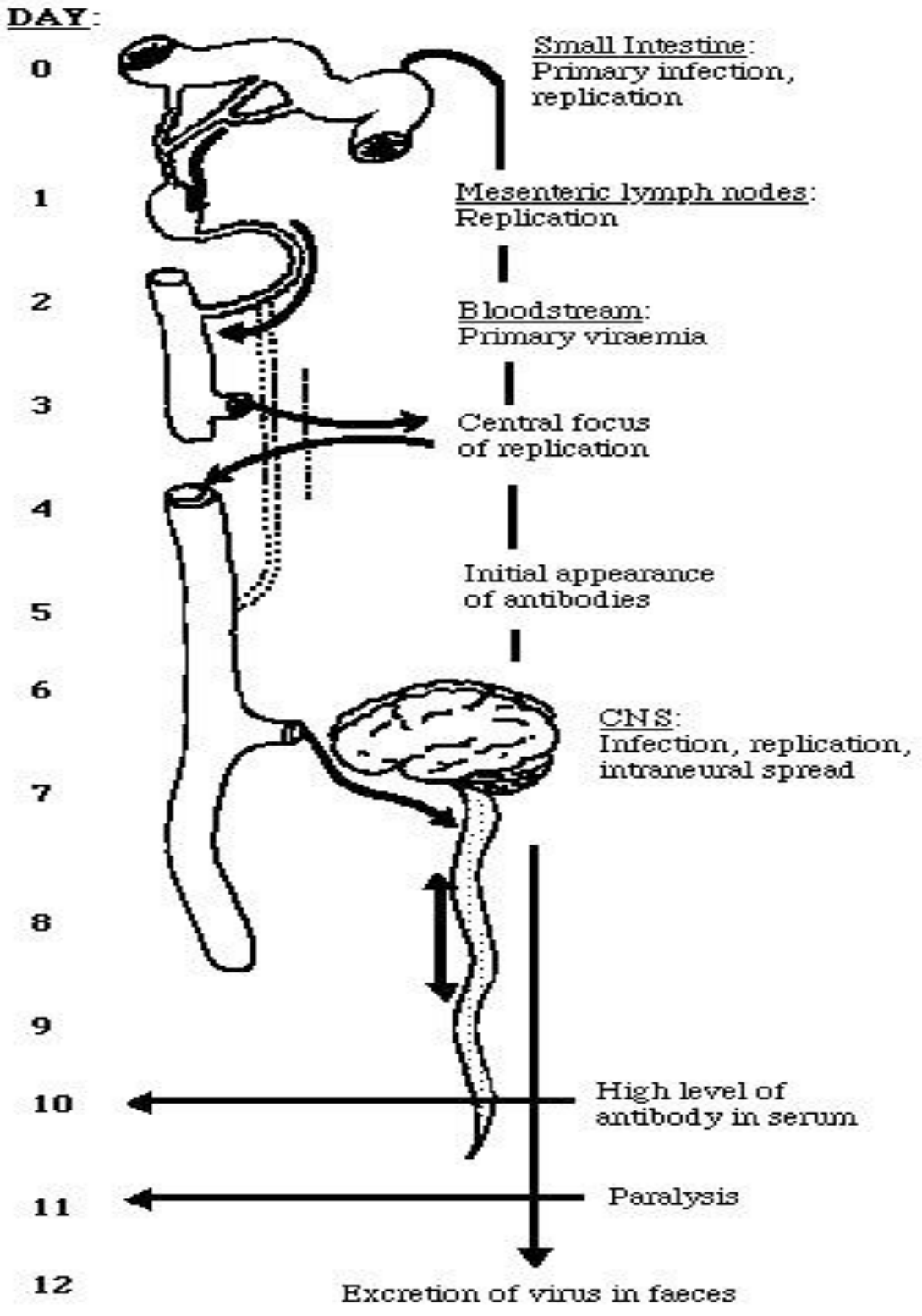


Figure 1.4: Bodian's classic diagram of polio pathogenesis

(http://www-micro.msb.le.ac.uk/3035/Picornaviruses.html)

1.1.4 Immunological responses

1.1.4.1 Innate immune response

When viruses invade the human body, host antiviral innate immune responses are activated through pathogen recognition receptors such as retinoic acid-inducible gene (RIG-I)-like receptors (RLRs) and Toll-like receptors (TLRs) [46] or NOD-like receptors (NLRs) [13] that respond viral RNA as an antigen. As a result, pathways of cytokine and chemokine production are triggered. Both type of receptors, TLRs and RLRs, modify the expression of hundreds of genes encoding for a diversity of cytokines, chemokines, and other proteins, some of which can directly counter virus infection, e.g. Protein Kinase regulated by RNA (PKR) and type I interferons (T1IFNs) response which play an important role in regulating infection by altering tissue tropism, while NLRs may regulate the development of the adaptive antiviral immune [13]. Those responses have been revealed in vitro in some studies, eg. CV-B4 triggers TLR4 on human pancreatic cells, or CV-B3 activates TLR4 knockout (KO) mice, resulting in reduced virus titers and myocarditis. Stimulation of TLRs may increase the severity of disease, e.g. TLR4 stimulants such as lipopolysaccharides (LPS) greatly increase the severity of CVB-induced myocarditis, or conversely may control CVB infections, eg. TLR3-triggered responses protect against myocarditis associated with CV-B3 [13].

Briefly, the innate immunity response help to start the nonspecific defense mechanisms immediately or within hours of an enterovirus antigen's appearance in the body, thence activating the system inflammation response and the adaptive immunity response.

1.1.4.2 Adaptive immune responses

Humoral immunity

The virus triggers an immunoglobulin M (IgM) response that can be detected by the second day and may persist for 3 months after the onset of symptoms [47, 48] as well as a strong neutralizing IgG response that recognizes epitopes in the N-terminal segment of VP1 [13]. While immunoglobulin A (IgA), which has been found in enterovirus infections [49], is responsible for maintaining the local immunity barrier.

IgA persists in the intestinal mucous for 2-4 weeks after infection, and protects the host from secondary infections [50] that can occur in situations where the enterovirus persists in the gut for many weeks after infection [38, 51]. Immunological responses to enterovirus infection are quite specific in terms of serology [52]. Therefore, patient may develop the symptoms if they are infected later with a different serotype [53], but are usually asymptomatic when re-infected with the same serotype.

Cell mediated immunity

Together with the humoral immune response, cell mediated responses provide a strong barrier to protect people from viral infections. Memory T-helper 1 (Th1) CD4+ T-cell responses specific for the three epitopes in VP1 have been identified in EV-A71-positive individuals [54]. CD4+ T-cells may help enhance the production of strong neutralizing antibody responses in mice infected with CV-B3 [55]. Another study showed that a decrease in different regulatory T-cell populations correlates with an increase in EV-A71 disease severity [56]. Studies in CV-B3 infected mice have show that myocarditis was related to cytotoxic T cell responses [57, 58]. B cells may act as a reservoir for virus dissemination during persistent CVB infection [59], and hereditary or acquired defects in B lymphocyte function, such as X-linked deficiencies, result in the persistent enterovirus infections [23, 60].

1.1.5 Clinical manifestations of non-polio enterovirus infections

With the success of the World Health Organization (WHO) international polio eradication program, clinical disease due to wild-type poliovirus is rarely seen nowadays [61]. However occasional vaccine-associated cases do occur following use of live attenuated polio vaccines; these cases are similar to wild-type infections but are rare. This section will therefore focus on the range of clinical manifestations seen with non-polio enteroviruses.

Among this group, i.e. the non-polio enteroviruses, the vast majority of infections are either asymptomatic or mild undifferentiated febrile illness. Typically there is sudden onset of high fever that may be accompanied by symptoms such as myalgia, headache,

sore throat, nausea, vomiting, mild abdominal discomfort, or diarrhea. The fever sometimes lasts up to a week and may show a biphasic pattern (Figure 1.5) [62].

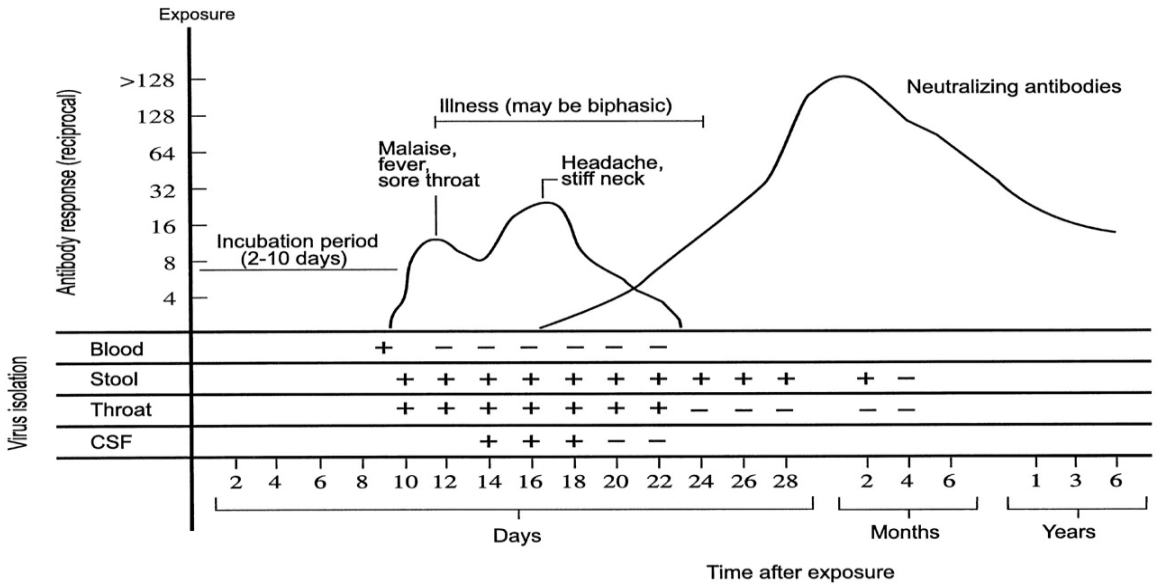


Figure 1.5: Clinical course, viral isolation, and antibody response in enteroviral infection

(Theoklis Zaoutis, and Joel D. Klein Pediatrics in Review 1998;19:183-191)

In the second phase of viral dissemination, enteroviruses can cause a wide range of signs and symptoms with a broad spectrum of severity, ranging from high fever to life-threatening manifestations. Clinical manifestations vary from asymptomatic disease to severe syndromes such as aseptic meningitis, myocarditis, and encephalitis (Table 1.1). These manifestations may occur alone or together [25, 63]. For example, EV-71 causes aseptic meningitis, encephalitis, HA, and lower respiratory tract infections. While EV68 infection in children may produce a respiratory outbreak characterized by pneumonia and wheezing [12]. These clinical signs and symptoms may be indicative of disease progression, that may be related to the virulence of the particular serotype, and/or the nature of the individuals immune response to infection [64].

Table 1.1: Clinical manifestations of non-polio enterovirus infections [8]

Clinical Syndrome	CV group A	CV group B	Echovirus	Enterovirus
Asymptomatic infection	All serotypes	All serotypes	All serotypes	All serotypes
Undifferentiated febrile illness	All serotypes	All serotypes	All serotypes	68, 70, 71
Aseptic meningitis (often associated with an exanthema)	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 14, 16, 17, 18, 22, 24	1, 2, 3, 4, 5, 6	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 14, 16, 17, 18, 20, 21, 22, 23, 24, 25, 30, 31, 33	70, 71
Encephalitis	2, 4, 5, 6, 7, 9, 10, 16	1, 2, 3, 4, 5	2, 3, 4, 6, 7, 9, 11, 14, 17, 18, 19, 22, 25, 30, 33	70, 71
Acute flaccid paralysis (Poliomyelitis-like)	4, 5, 6, 7, 8, 9, 10, 11, 14, 16, 21, 24	1, 2, 3, 4, 5, 6	1, 2, 4, 6, 7, 9, 11, 14, 16, 17, 18, 19, 30	
Myopericarditis	1, 2, 4, 5, 7, 8, 9, 14, 16	1, 2, 3, 4, 5, 6	1, 2, 3, 4, 6, 7, 8, 9, 11, 14, 16, 17, 18, 22, 25, 30	
Pleurodynia	1, 2, 4, 6, 9, 10, 16	1, 2, 3, 4, 5, 6	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 14, 16, 19, 22, 25, 30	
Herpangina	1, 2,3, 4, 5, 6, 7, 8, 9, 10, 16, 22	1, 2, 3, 4, 5	6, 9, 11, 16, 17, 22, 25	71
Hand-foot-and-mouth disease	4, 5, 7, 9, 10, 16		7	71
Exanthems	2, 4, 5, 7, 9, 10, 16	1, 2, 3, 4, 5	2, 4, 6, 9, 11, 16, 18, 25	
Common cold	2, 10, 21, 24	1, 2, 3, 4, 5	2, 4, 8, 9, 11, 20, 25	
Lower respiratory tract infections	7, 9, 16	1, 2, 3, 4, 5	4, 8, 9, 11, 12, 14, 19, 20, 21, 25, 30	68, 71
Acute hemorrhagic conjunctivitis	24			70
Generalized disease of the newborn	3, 9, 16	1, 2, 3, 4, 5	3, 4, 6, 7, 9, 11, 12, 14, 17, 18, 19, 20	

Aseptic meningitis: CV group B viruses and Echovirus are usually the main causes of aseptic meningitis. Outbreaks usually associated with single serotype of echovirus occur every 5-12 years.

Prodomal symptoms include fever, sore throat and headache. Meningitic signs vary from minor to severe, with Kernig's sign and Brudzinski's sign present in one-third of patients. The cell count in cerebrospinal fluid (CSF) is usually abnormal, with counts of 10 to 500 cells/mm^3, sometimes up to1000 cells/mm^3 early in infection. At this time neutrophil counts may be dominant later switching to lymphocytes after a few days. Occasionally cell counts may be as low as 10 cells/mm^3. Glucose level is normal and proteins are also either normal or only slightly increased in CSF. Enterovirus may be identified in CSF or other samples using PCR or cell culture methods. In suspected enterovirus meningitis cases, the sensitivity of PCR ranges from 66 to 90% on CSF, while the sensitivity of viral isolation ranges from 30 to 35%. But when combined with culture of other specimens such as throat swab, urine, faeces, the ability to detect virus increases.

Admission is not necessary for all patients with aseptic meningitis. However, hospitalization is recommendation if patients have confusion, muscle weakness, or rash, since these signs may indicate that disease could progress to severe disease.

Encephalitis: Enteroviruses, including poliovirus, accounting for 11-22% of virally defined encephalitis cases and 5% of clinically diagnosed cases. Thus enteroviruses rank fourth after other agents causing encephalitis, such as arboviruses, herpes simplex, and lymphocytic choriomeningitis virus [65, 66]. However, during epidemics this proportion is likely to rise higher. According to Cheng el al (2008), enterovirus was the cause of 24 % of encephalitis cases in Taiwan [63]. Many serotypes can cause encephalitis (Table 1.1). In some studies of HFMD in the Asia Pacific region, EV-A71 was prominent in the etiology of a range of CNS manifestations including encephalitis [67, 68].

Encephalitis is just one of several clinical features of systemic enterovirus infection in neonate. However, in older children and adolescents the signs and symptoms of

enteroviral infection are usually limited to the CNS. Clinical manifestations vary from stupor, lethargy, and behavioral abnormalities to convulsions, paralysis and coma. Focal encephalitis in children presents with localized seizures, abnormal movements, and/or ataxia that can mimic herpes simplex encephalitis.

Most neonates with encephalitis caused by CV and Echovirus recover fully. Some cases have persistent neurological sequelae, but fatalities are rare. In contrast encephalitis that is associated with EV-A71 or echovirus 7 often presents a more complicated clinical picture. However the most concerned feature is brainstem encephalitis, since ANS dysregulation may happen, potentially with rapid progression to cardiopulmonary collapse and a fatal outcome [69, 70].

Acute flaccid paralysis (AFP): Poliomyelitis, resulting in paralysis or weakness of one or more limbs has been virtually eradicated globally, except for rare reports from some developing countries. However, acute paralysis similar to paralytic poliomyelitis can occur with other serotypes of enterovirus, particularly EV-A71 and EV-D68; such complications have been described in outbreaks in America and Russia [71-73]. Recently, AFP was reported in about 10-15% of cases who had neurological manifestations during and HFMD outbreak in Taiwan, China [74, 75]. Huang reported that 4 patients had acute unilateral or bilateral flaccid paralysis with or without other signs of HFMD. One patient presented with a transiently atonic neurogenic bladder. Three of the four patients had abnormal lesions of the left anterior horn or ventral roots on Magnetic Resonance Imaging (MRI).

Other clinical manifestations: Enterovirus species can also cause a range of other rare clinical features, such as myopericarditis, which is mostly associated with CV-B3 [76, 77], or acute hemorrhagic conjunctivitis, which is usually associated with EV-A70 [14] or CV-B24 [78] (Table 1.1). Recently, EV-D68 was responsible for several outbreaks of lower respiratory tract disease in China [11], Japan [79] and some Western countries [10, 80].

1.2 Hand, Foot and Mouth Disease (HFMD)

HFMD had been described as a clinical syndrome since 1928 [81]. This disease is viewed as one of the common clinical manifestations of enterovirus infection and is usually self-limited. CV-A16 is a common causative agent, but many other enteroviruses, including EV-A71, can cause this disease. During the last two decades, a small proportion of HFMD patients have been noted to progress rapidly to severe and sometimes fatal outcomes. The spectrum of clinical features of HFMD has expanded considerably, and now includes minor conditions such as HA through to very severe disease, such as brainstem encephalitis, myocarditis, and pulmonary edema that may be fatal [64, 82, 83].

Children under 10 years old are usually the infectious source to other family members. Patients often complain of sore throat and anorexia, and after 2 days of fever may specifically present with small vesicles in the cheek, buccal mucosa and tongue. These lesions can coalesce into larger vesicles and break down to form the typical ulcers that are found when examining the mouth. Approximately 75% of cases present with specific vesicles on the palms, soles, buttocks, and also sometimes the elbows, knees, and genitalia. Skin biopsy of these lesions reveals inflammation in the dermis layer accompanied by infiltration of lymphocytes and neutrophils around small vessels in the epidermis layer.

1.2.1 Global Epidemiology of HFMD

HFMD has been a common, relatively mild pediatric disease in North America and Western Europe for many years. However, this epidemiology have changed recently [84]. In particular outbreaks of EV-A71 associated disease have been noted in the Asia Pacific region for the last 3 decades. In 1987, a serious outbreak of HFMD was reported in Hong Kong, caused by EV-A71 [85]. Then in 1997, EV-A71 was reported as the cause of other HFMD outbreaks in Southeast Asia such as Sarawak, Malaysia [86, 87]. In 1998, an EV-A71 outbreak was reported in Taiwan affecting 125,000 children, with 405 of them being very severe, particularly children under 5 years old. EV-A71 was isolated in 92% of the 78 deaths [88]. EV-A71 was the causative in fatal cases associated with

HFMD in Ho Chi Minh City, Vietnam in 2003 [89, 90]. From then onwards, HFMD has spread to nearly all the Asia-Pacific region (Figure 1.6).

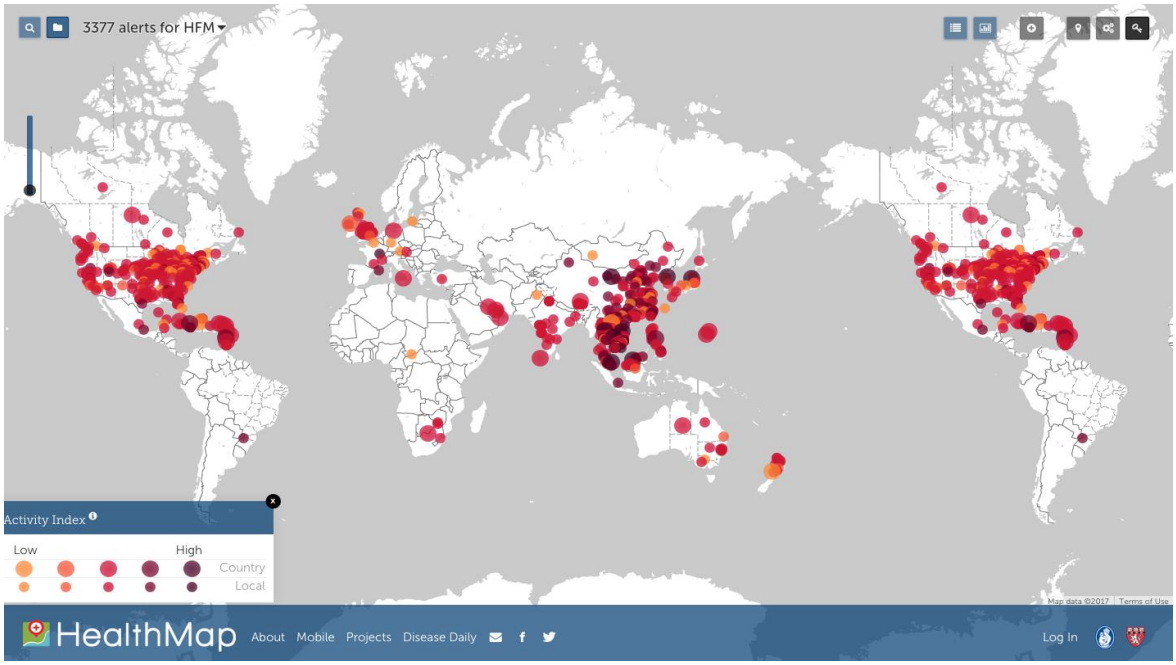


Figure 1.6: Distribution of HFMD around the world (updated from Health Map in May 2017). (http://www.healthmap.org)

HFMD is now endemic in many places but with increasing numbers of cases seen in summer and autumn [28]. All age groups are affected but infection rates are higher in children under 6 years old, especially infants, in whom infection rates are several times higher than in older children or adults [91-94]. For unknown reasons, males seem to have about 50% higher risk of infection than females. In one genetic study in Taiwan with different distribution of gender, HLA-A33 was related to host susceptibility to EV-A71, but it did not show the link between this factor and gender [95].

1.2.2 Epidemiology of HFMD in Viet Nam

HFMD was first reported in Viet Nam in 2003 following a series of deaths in small children under 3 years of age who presented with features of HFMD and deteriorated rapidly within 24 hours of admission [96]. EV-A71 was identified as the causal agent in these cases [90]. Initially HFMD cases were noted mostly in Ho Chi Minh City, but by 2005 the disease had spread to many provinces across southern Viet Nam [89]. By

2008, in addition to outbreaks occurring in all provinces in southern Viet Nam, HFMD was also noted among children living in the central and northern regions (Vietnam CDC data, unpublished). Then, in a major outbreak in 2011, 164 deaths were reported among 106,000 children who were diagnosed clinically with HFMD across all provinces [97] (Figure 1.7). Later, in the HFMD outbreak of 2011-2012, about 10% of the encephalitis cases associated with HFMD were found to be infected by other non EV-A71 enterovirus serotypes (unpublished data).

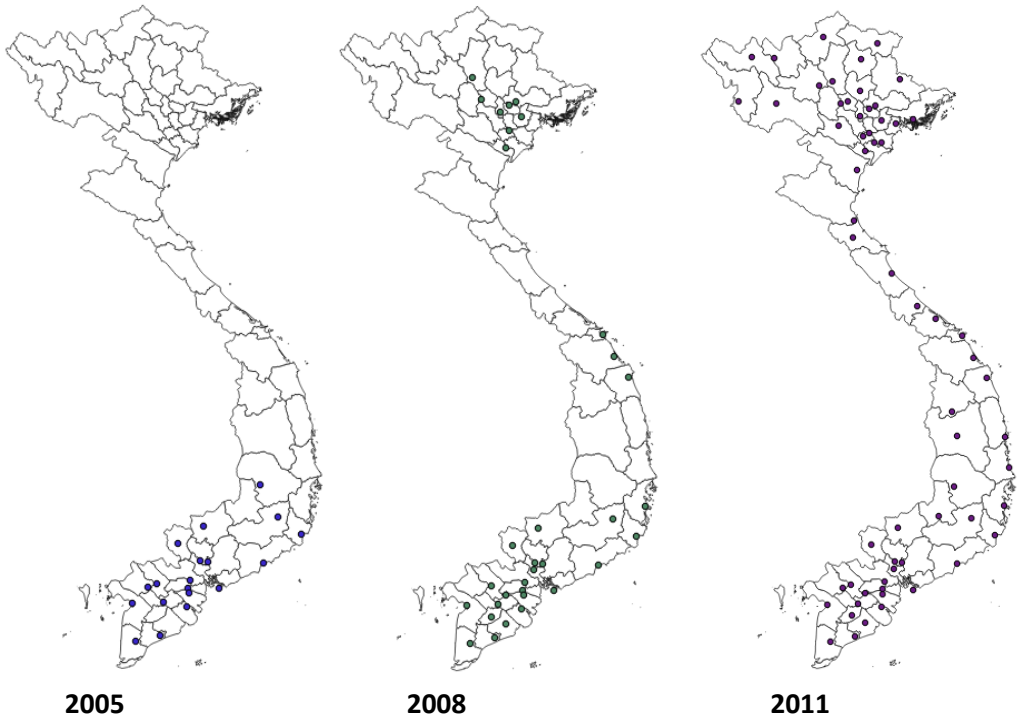


Figure 1.7: Maps of HFMD spreading in Viet Nam from 2005 to 2011

(based on data from Pasteur Institute [89], ProMEDmail sources, and Vietnam CDC (unpublished).

According to 2011-2014 surveillance reports (Vietnam CDC data, unpublished), HFMD continued to occur endemically and was considered a common disease, with incidence rates varying from 76 to 171 cases, and mortality rates from 0.01 to 0.19 cases, per 100.000 population [98].

1.2.3 Clinical features

HFMD: Most cases present with skin lesions or mouth ulcers only. Approximately 10-30% of hospitalized cases progress to CNS complications, including meningitis, polio-like paralysis, brainstem encephalitis, etc., in young children [68]. In some cases CNS complications, i.e. brainstem encephalitis, progress rapidly to pulmonary edema and cardiac failure and death finally. Most of these severe cases are infants or young children [69]. The clinical features vary depending on the main serotype circulating, the virulence of the virus, the nature of the host's immune response, and the presence of co-infections (Table 1.1).

The prodromal symptoms include low-grade fever and malaise, followed by papulovesicular rash involving the palms or soles of the feet and sometimes knees, elbows, and buttocks, often with mouth ulcers. Other dermatological manifestations include peri-oral rash and onychomadesis [99]. Recently, some reports have suggested that HFMD related to CV-A6 has a more widespread, severe, and varied rash than classic HFMD. In these cases a maculopapular rash with or without vesicles may appear on the buttocks, knees or elbows, and trunk, and needs to be distinguished from bullous impetigo, eczema herpeticum, vasculitis, and primary immunobullous disorders [99-101].

Picture 1: Vesicles on the palm and maculopapular rash on the knee of HFMD patients

Table 1.2: Proposed clinical case definitions for HFMD/HA and associated complications (WHO) [83]

Disease	Proposed Case Definition
HFMD	Febrile illness with papulovesicular rash on palms and soles, with or without vesicles/ulcers in the mouth
HA	Febrile illness with multiple oral ulcers on the posterior parts of the oral cavity.
Aseptic meningitis	Febrile illness with headache, vomiting and meningism associated with presence of more than 5 – 10 white cells per cubic millimeter in cerebrospinal (CSF) fluid, and negative results on CSF bacterial culture.
Brainstem encephalitis	Myoclonus, ataxia, nystagmus, oculomotor palsies, and bulbar palsy in various combinations, with or without MRI. In resource-limited settings, the diagnosis of brainstem encephalitis can be made in children with frequent myoclonic jerks and CSF pleocytosis.
Encephalitis	Impaired consciousness, including lethargy, drowsiness or coma, or seizures or myoclonus.
Encephalomyelitis	Acute onset of hyporeflexic flaccid muscle weakness with myoclonus, ataxia, nystagmus, oculomotor palsies and bulbar palsy in various combinations.
AFP	Acute onset of flaccid muscle weakness and lack of reflexes.
Autonomic nervous system (ANS) dysregulation	Presence of cold sweating, mottled skin, tachycardia, tachypnea, and hypertension.
Pulmonary edema/ haemorrhage	Respiratory distress with tachycardia, tachypnea, rales, and pink frothy secretion that develops after ANS dysregulation, together with a chest radiograph that shows bilateral pulmonary infiltrates without cardiomegaly.
Cardiorespiratory failure	Presence of tachycardia, respiratory distress, pulmonary edema, poor peripheral perfusion requiring inotropes, pulmonary congestion on chest radiography and reduced cardiac contractility on echocardiography.

HA: is another manifestation of HFMD that can present without skin symptoms. Typically, children present first with multiple, painful mouth ulcers in the anterior pharyngeal folds, uvula, tonsils and soft palate, though sometimes these lesions can be found on the buccal mucosa, tongue and lips as well. The children usually refuse to eat or drink because of the painful oral ulcers. Typical skin lesions may then develop on their palms and soles a day or two later

HFMD and HA can be seen separately or together during outbreaks, although one clinical presentation usually predominates. Both HFMD and HA are caused by HEV-A, including Coxsackie A (serotypes 4, 5, 6, 7, 9, 10, 16) and EV-A71. HA are also caused by other serotypes of Coxsackie A, Coxsackie B, and Echovirus (Table 1.1). Co-circulation of these serotypes during epidemics of HFMD/HA may result in clinically indistinguishable mucocutaneous lesions. Aside from the possibility of progression to severe disease (see below) the most common clinical problem occurring with HFMD/HA patients is dehydration because of inadequate intake of fluid due to the painful mouth ulcers.

1.2.3.1 Brainstem encephalitis

Brainstem encephalitis associated with EV-A71 presents with specific clinical manifestations. These include myoclonic jerking, vomiting, ataxia, nystagmus and cranial nerve palsies. According to Lai (1997), “myoclonus’’ is a sudden, brief and involuntary muscular contraction that can occur unilaterally or bilaterally and may be symmetrical or asymmetrical, involving the head, neck, trunk, and limbs. Myoclonic jerks can occur when the central nervous system, especially the brainstem and forebrain, is damaged [102]. Myoclonic jerks have been reported variously in 22-68% of hospitalized HFMD patients, and in 86% of patients with brainstem encephalitis they are associated with severe disease and a higher incidence of neurological sequelae [74, 103, 104]. Therefore, the occurrence of myoclonic jerk in HFMD patients is considered to be evidence of CNS involvement and one of the warning signs for progression to a more severe condition [83]. Other symptoms such as nystagmus, ataxia and tremor are said to occur in about 60% of HFMD cases with associated brainstem encephalitis, and

may indicate involvement of particular regions of the brainstem. Involvement of cranial nerve nuclei may results in the cranial nerve palsies that can be seen in 27% of the brainstem encephalitis [74]. Respiratory distress, including hyperventilation and/or irregular respiratory patterns such as Cheyne-Stokes breathing, occurs in about 22% and these disorders are associated with the severity of disease [74].

1.2.3.2 Autonomic nervous system (ANS) dysregulation

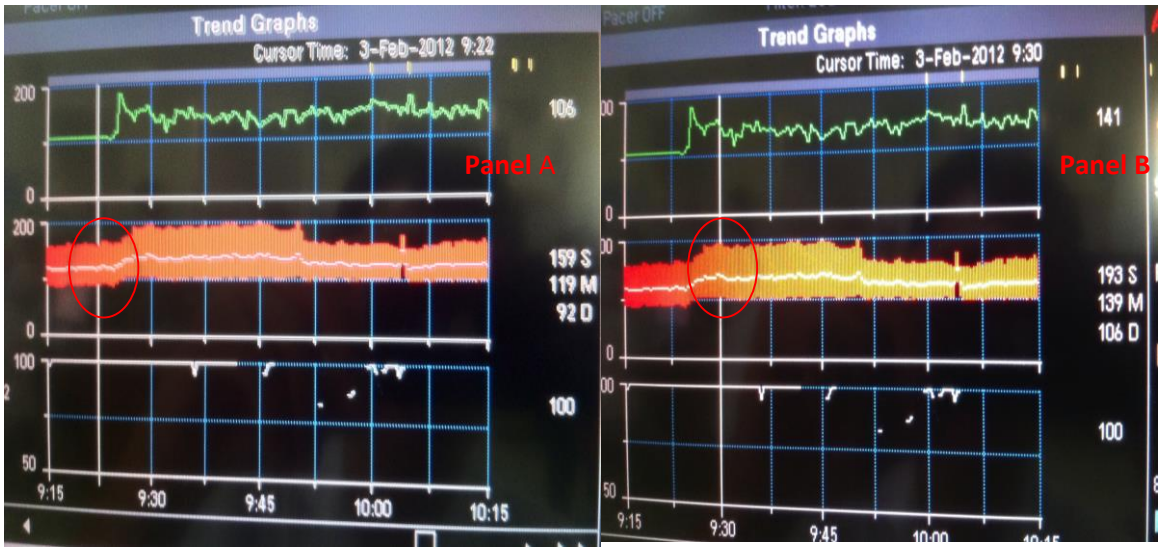
Dysregulation of the ANS pathways can progress to increasingly severe clinical symptoms, including non-cardiogenic pulmonary edema, which can rapidly result in cardiorespiratory failure and death.

Since many of the signs are rather non-specific and can be present anyway among children with high fever, or may mimic the normal physiological responses in young children in whom both the peripheral and central nervous systems are developing, the frequency of ANS dysregulation in severe HFMD is not clear. However, the emergence of systemic hypertension (HTN) in HFMD patients is general acknowledged as clear evidence of ANS dysregulation. The proportion of children with hypertension among HFMD cases is also not clear however, since only severe cases are usually reported; more than 36% developed systemic hypertension in a study of 36 severe EV-A71 infection cases from Taiwan [105], while 17 patients presented with hypertension in 22 severe HFMD cases reported from China [106]

Tachycardia is another feature of ANS dysfunction and may present at an earlier stage. In clinical practice, a heart rate (HR) persistently over 150 beats/min in the resting condition and adjusted for fever is considered as one indicator of ANS dysfunction [83]. Cardiovagal HR tests and assessment of heart rate variability (HRV) have been suggested as ways to diagnose and monitor autonomic dysfunction [107-109], but these tests are not yet practical options in most areas where severe HFMD is an issue. In genera HRV decreases in severe HFMD and may associate with CNS involvement and predict cardiopulmonary collapse [110].

Mottled skin reflects vasoconstriction, and was noted to occur in 7 patients among the

29 fatal cases described in the Sarawak outbreak [70]. Profuse sweating has also been reported in severe HFMD [111]. Other signs and symptoms of ANS dysregulation include refractory fever, i.e. core temperature > 40°C for at least 4 hours despite antipyretics, and hyperglycemia [112]. These signs may be warning signs of likely progression to severe disease [83]. Patients who present these features suggestive of hyper-activation of the sympathetic system could progress to heart failure after 2-5 hours [111].



Picture 2: rapidly increasing blood pressure from 159/119 mmHg (Panel A) to 193/139 mmHg (Panel B) after about 8 minutes in a 2 year old Vietnamese child with HFMD managed on PICU during the 2011-2012 outbreak

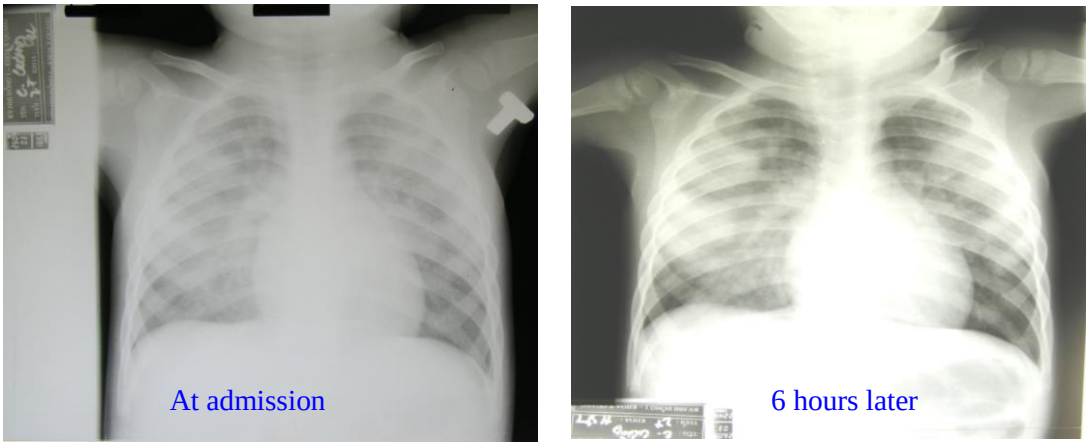
Tachypnea and irregular breathing are also considered as features of ANS dysregulation but they may also be related due to brainstem involvement. Some patients with these symptoms progress rapidly to more severe respiratory distress, including stridor, respiratory muscle retractions, Cheyne-Stokes breathing, etc. indicating laryngeal/respiratory muscle paralysis, and potentially development of pulmonary edema or cardiorespiratory failure.

1.2.3.3 Pulmonary edema and cardiorespiratory failure.

Patients with pulmonary edema usually develop respiratory distress, i.e. increased work of breathing with frothy pink secretions often requiring intubation and

ventilation. Typically, this occurs in patients with brainstem encephalitis patients who present initially with tachypnea, then deteriorate rapidly over a short period while under observation, developing increasingly severe respiratory breathing, tachycardia and hypertension. But sometimes, patients presenting with severe respiratory distress at admission and suffering from fulminant pulmonary edema may get even worse, going on to develop hypotensive shock with poor cardiac output. These patients with cardiorespiratory failure are hypotensive requiring inotropes and have poor peripheral perfusion, with evidence of decreased cardiac contractility on heart ultrasound and pulmonary congestion without cardiomegaly on chest X-ray.

Some studies describe that the acute cardiorespiratory deterioration is characterized by tachycardia, tachypnea, increased respiratory effort, central fever, and poor perfusion [113-115]. Most of patients described were also hypertensive[67]. In a study to understand the mechanism underlying fulminant pulmonary edema, the mean arterial pressure (MAP) dramatically increased and fluctuated before the onset of pulmonary edema and then dropped subsequently to hypotensive levels before development of cardiorespiratory failure and death [114]. In that study the time from onset of respiratory distress to death was about 4 hours.



Picture 3: Pulmonary edema in a 2yr boy diagnosed with enterovirus associated encephalitis: X-rays at admission and 6 hours later (unpublished, 2003)

The proportion of patients developing pulmonary edema has varied from 24 to 72 % of fatal cases [70, 97]. In addition refractory shock or respiratory failure or a combination

of these factors were thought to be responsible for more than 80% of deaths in one series, of whom 69% died of refractory shock [97].

1.2.4 Pathogenesis and pathophysiology

To date there is no good evidence to confirm that enteroviruses invade the lungs and heart directly as targets. Research has not revealed histological or virological evidence of direct myocardial invasion in patients with EV-A71 associated brainstem encephalitis/hypertension who had left ventricular dysfunction [111]. However, there is evidence that enteroviruses, especially EV-A71, can invade and spread within the CNS, including the brainstem. The impact of altered cytokine profiles on the cardiopulmonary system may also influence the severity of HFMD [116].

1.2.4.1 Systemic inflammatory responses in HFMD

System inflammatory responses to enteroviruses, particularly EV- A71, may produce a cytokine storm. This phenomenon is thought to play an important role in the pathogenesis of severe HFMD, including in development of pulmonary edema, myocarditis and encephalitis [117, 118]. EV-A71 related HFMD disease severity has been found to relate to altered levels of a number of different cytokines, including TNF∝, INFγ, IL6, and IL13, in both blood and CSF, with evidence that these concentrations correlate not only with the degree of brainstem and spinal ganglia involvement [119] but also with signs of enhanced vasomotor and cardiac sympathetic drive [120]. The levels of various pro-inflammatory cytokines were significantly higher in patients who manifested acute respiratory failure [116, 121, 122]. This increase in pro-inflammatory cytokines may lead to increased vascular permeability in the lung, altering the integrity of this barrier to fluid and protein flow into the lung alveolar and interstitial spaces, and thus contributing to the non-cardiogenic pulmonary edema [123]. Concentrations of many cytokines (interleukin (IL) 1b, IL 1b, IL-6, and IL-8) and chemokines (IP-10, MCP-1, and G-CSF) are increased in the CSF in patients with encephalitis [122]. However, interestingly GM-CSF, MIP-1**b**, IL-2, IL-33, and IL-23, IL-3, IL-6, IL-12p40, and tumor necrosis factor (TNF)-**a** were also elevated in uncomplicated HFMD cases [122, 124]. Therefore, at this time the influence of cytokines and

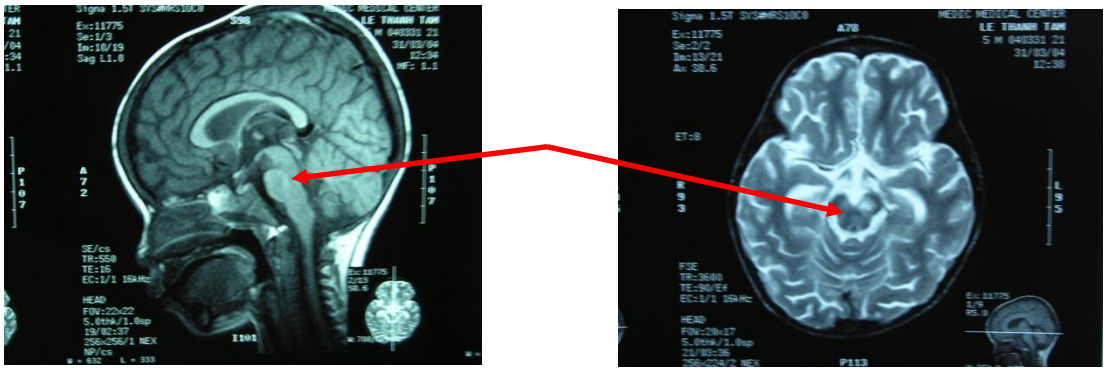
chemokines on disease severity remains to be clearly elucidated.

Host inflammatory responses are also considered to influence the thrombocytosis, neutrophilia and hyperglycaemia that can occur in severe HFMD patients, with these factors considered as risk factors for severe HFMD [125].

1.2.4.2 Central nervous system involvement – neurotropism and pathology

Different enterovirus serotypes exhibit different neurotropic responses. A model using neonatal mice with CV-B3 infection showed that the virus was expressed in the choroid plexus and the subventricular area [126]. Moreover, CV-B also damaged the cortex and the hippocampus regions in another experiment in an animal model [127]. Echovirus 1 may cause lesions in the motor cortices in infected animals resulting in paralysis of the hind limbs [128]. Meanwhile, EV-A71 attacks the spinal cord, cerebellum, medulla oblongata, pons, brain stem, midbrain, dentate nuclei, and cortex according both to clinical observational studies and an MR imaging study [74, 129, 130]. At autopsy deterioration of neurons was shown, with invasion of monocytes into the gray matter of the pons and spinal cord, and EV-A71 was isolated in brainstem tissue and CSF [70].

Brain CT and MRI have shown damage to the brainstem in encephalitis associated with EV [130-133]. In particular MRI showed damage to the pons in the midbrain and the dentate nucleus in the cerebellum. Damage was also seen in cervical spinal cord in some cases, although these lesions resolved on scans taken 2 weeks to 2 months later [132].



Picture 4: MRI showing brainstem involvement in a 5 month old infant presenting with neurological complications (unpublished data, 2004).

According to Plum and Posner (1982), patients with brainstem damage or herniation usually present abnormalities in respiratory pattern and pupil responses. Damage in the hippus leads to dilated and fixed pupils; damage to the 3rd cranial nerve causes asymmetrical dilated pupils; damage to the pons leads to pinpoint pupils; while lesions in the midbrain lead to mid-position fixed pupils. Hyperventilation, Kussmaul's breathing, irregular breathing, Cheyne-Stoke's or gasping respirations also indicate damage to the patient's brainstem (Figure 1.8) [134].

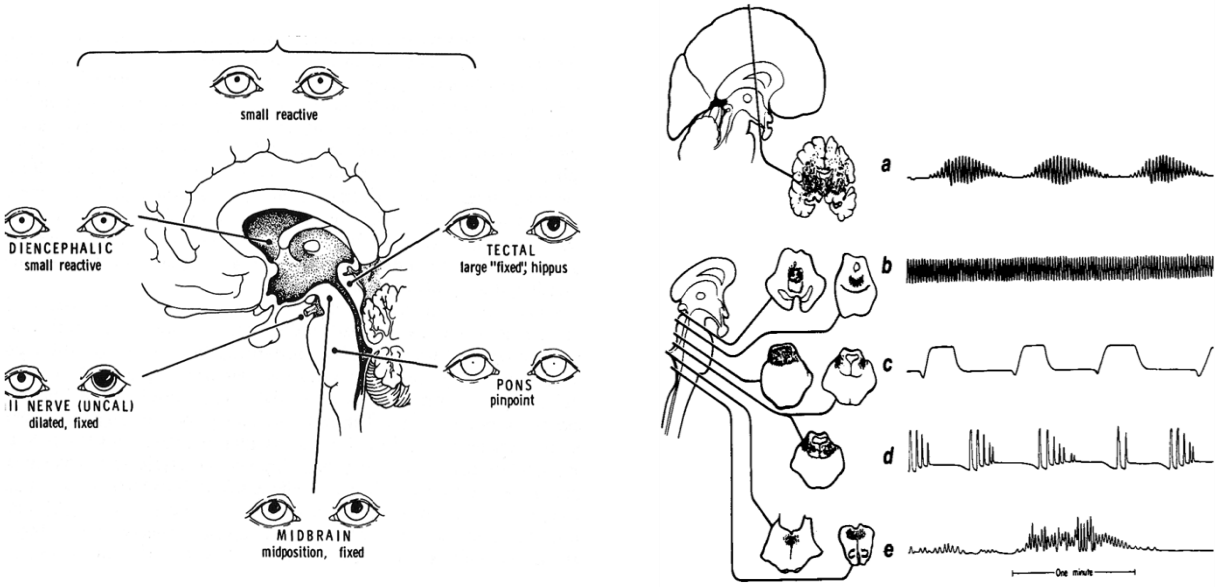


Figure 1.8: Respiratory patterns and pupil abnormalities associated with brainstem damage [134].

1.2.4.3 ANS dysregulation

The ANS is a visceral and largely involuntary motor/effector system including sympathetic and parasympathetic parts, each with a central and a peripheral component. However, the diverse functions of the peripheral ANS are integrated and regulated by the central ANS, the extensive circuitry of which ranges from the forebrain to the brainstem (Table 1.3) [135].

Although the mechanisms underlying the ANS dysregulation are not yet understood for HFMD, there is evidence that inflammation occurs in the brainstem and spinal ganglia and

Table 1.3: Central ANS and Function

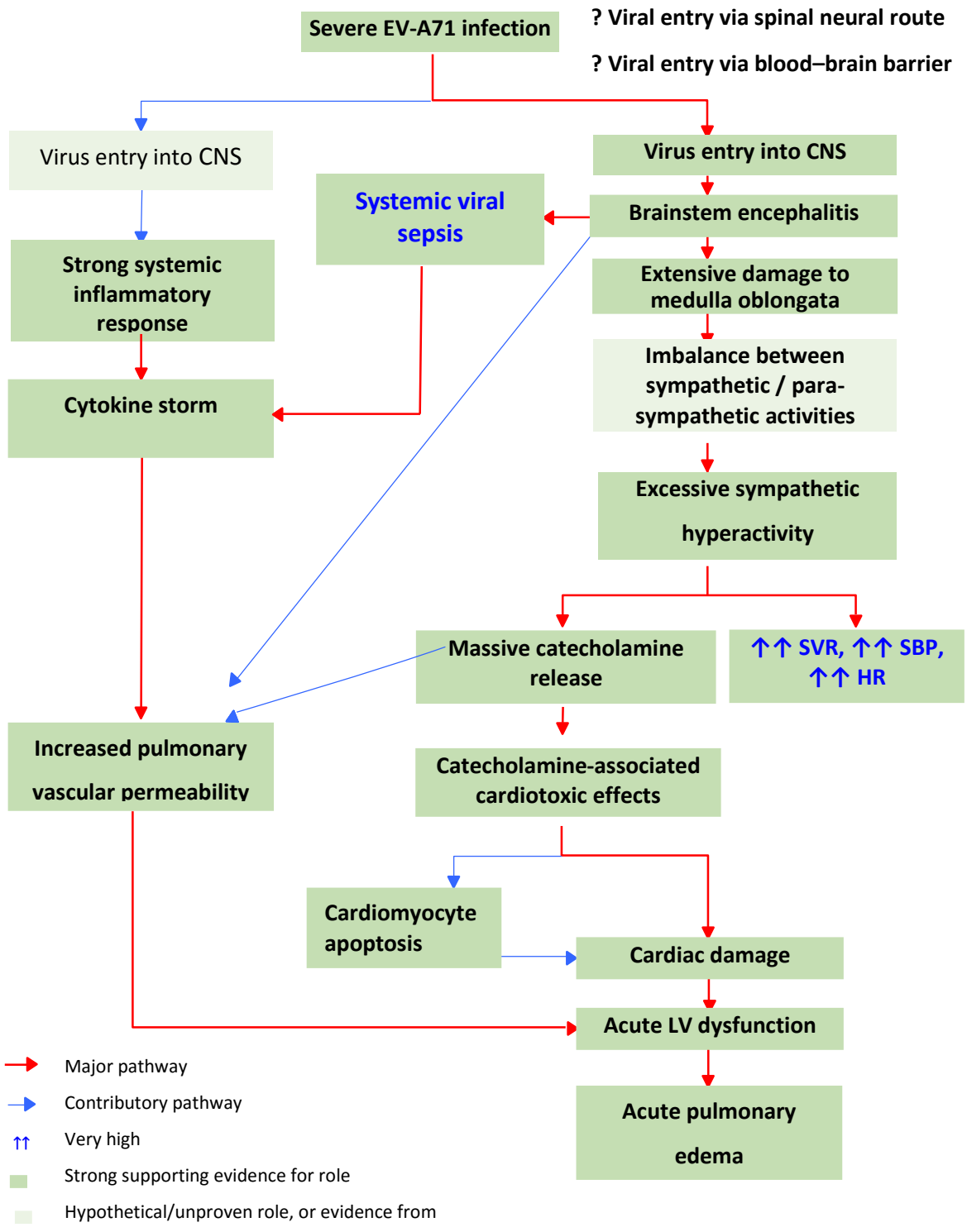
Anatomic Area	General Function	Clinical Manifestations
Insular and medial prefrontal cortices	High-order autonomic control: input from gastric mechanoreceptors, arterial chemoreceptors, baroreceptors	Cardiac arrhythmia
Extended amygdala	Autonomic expression of emotional states: integrates autonomic and motor responses	Viscerosensory phenomena (e.g., unilateral hyperhidrosis) Vomiting (left temporal focus) Sexual arousal
Hypothalamus	Homeostasis: initiates and coordinates biological rhythms, Autonomic, neuroendocrine, and behavioral responses	Hypothermia or hyperthermia Poor stress response (autonomic storm) Insomnia
Midbrain	Coordinates autonomic, pain-controlling, and motor mechanisms for stress-related, aggressive, and Reproductive behaviors	Hypertension or hypotension, arrhythmias Intractable vomiting and dysmotility Hypoventilation Urinary retention
Pons	Relays viscerosensory information to forebrain	
Nucleus of the tractus solitarius	Relays viscerosensory information from vagus and glossopharyngeal nerves to other Central ANS regions	
Medulla	Cardiovascular and respiratory control via premotor autonomic and respiratory neurons controlling input to spinal, respiratory, and preganglionic motor neurons	Sleep-disordered breathing (e.g., apnea, alveolar hypoventilation

that this damage correlates with cytokine profiles [119]. Increased sympathetic activity results in tachycardia, increased peripheral vascular system resistance, severe systemic and pulmonary hypertension, and eventually pulmonary edema [64, 120].

1.2.4.4 Pulmonary edema and cardiorespiratory failure

Pulmonary edema is often considered as the natural progression of ANS dysregulation resulting from excessive sympathetic hyperactivity in brainstem encephalitis. High plasma catecholamine concentrations were found in plasma from EV-A71 associated brainstem encephalitis/hypertension patients who had recently progressed to pulmonary edema [111]. Left ventricular failure in these patients could be related to catecholamine-associated cardiotoxic effects, which were found on histological examination of cardiac ventricular biopsies [64]. Clinical data describing HFMD patients who presented with hypertension before rapidly progressing to cardiorespiratory failure suggest that the excessive release of catecholamine could result in the hypertensive crisis that are similar to the emergency autonomic dysreflexia in spinal cord injury patients [105, 136, 137]. This may contribute to acute cardiorespiratory failure in the final stage of disease.

Therefore, the combination of acute heart failure and increased vascular permeability in lung alveolar may enhance the possibility of pulmonary edema in severe HFMD as the result of the entire process of pathophysiology of severe HFMD (Figure 1. 9).



EV-A71=enterovirus 71. CNS=central nervous system. SVR=systemic vascular resistance. SBP=systemic blood pressure. HR=heart rate. LV=left ventricular.

Figure 1.9: The postulated pathogenesis of enterovirus-71-associated acute pulmonary edema (modified from [64])

1.2.5 Laboratory diagnostics for enterovirus infection

1.2.5.1 Reverse transcriptase polymerase chain reaction

Detection of enteroviral nucleic acid in clinical specimens is the most widely used approach for diagnosis of HFMD. The method is sensitive, and suitable screening for high throughput of large numbers of samples. Overall, molecular methods are more sensitive than virus culture. In some studies the diagnostic yields by molecular assay and virus isolation were 66-86% [138, 139] and 26-34% [140-142], respectively.

Generic real time reverse transcription polymerase chain reaction (RT-PCR) targeting the 5'UTR, a highly conserved region across enterovirus genomes, can be used to generically detect enterovirus RNA in test specimens. For detection or identification of specific enterovirus serotypes, RT-PCR assays targeting more variable genomic regions such as VP1 are often employed. Currently, several serotype specific assays have been developed for EV-A71, CV-A16 and other HFMD associated CV-As. Additionally, serotype determination can be achieved by utilizing generic primers to amplify complete or partial VP1 region, followed by sequencing of the PCR amplification obtained.

1.2.5.2 Serology

There are a number of serological methods for diagnosis of enteroviral infections. These methods can be used to detect viral antigen directly, e.g. immunosorbent assays, or alternatively to identify antibodies that react to the viral antigen, eg. haemagglutination inhibition tests, complement fixation tests and neutralization tests. However these test are rarely used now, as molecular methods are preferred.

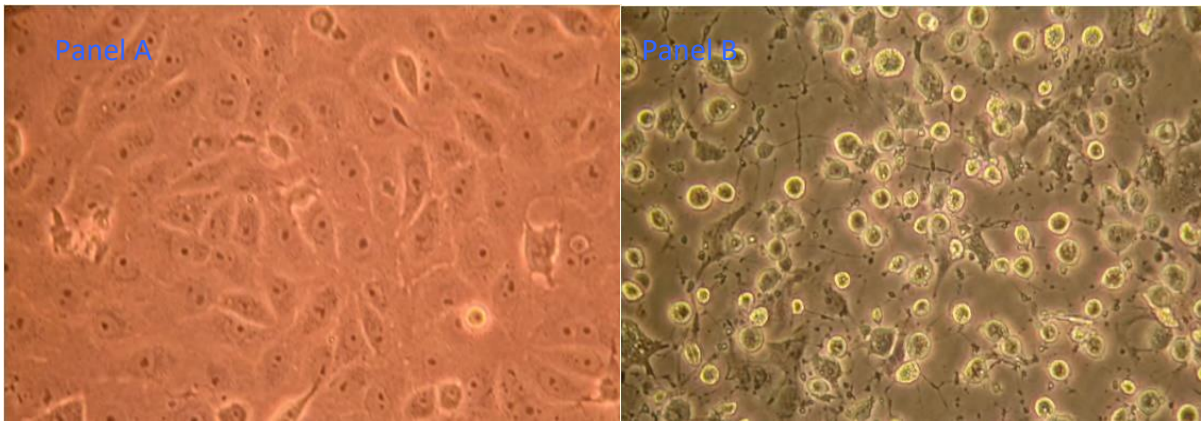
Microneutrolization tests are the most specific method to detect antibodies to enterovirus. However use of these tests is limited in routine practice because of poor sensitivity, and difficulties with standardization.

Serotype specific immunoassays are commercial serological methods used to detect antibodies to a range of serotypes including CV-B serotypes 1-6, a few echoviruses, and one or two CV-A serotypes. Cross reactivity is a problem however; using IgM ELISA

methodology 67% of CV group B infections were positive in the early stage of disease, but the results were not specific and could also be found with other enteroviral serotypes [143, 144]. IgM antibodies can persist for up to 6 months and IgG antibodies persist for years after infection, so both acute and recovery phase samples are needed, demonstrating a four-fold increase in antibody titers to verify the diagnosis of acute infection. As a result serological methods are not generally applied for acute HFMD diagnosis, but can be used for epidemiological surveillance.

1.2.5.3 Viral isolation

HFMD associated enteroviruses can be cultured in a wide range of cell lines such as RD (human rhabdomyosarcoma), LLC-MK-2 (Rhesus monkey kidney), Vero (African green monkey kidney), or BHK-21 (Baby Hamster Kidney). After inoculation, the cell cultures are observed daily for cytopathic effects (CPE), which may occur 2-6 days or longer after infection. Sometimes several passages (i.e. re-inoculation of cultured material in fresh cell lines) are required to maximize the sensitivity. Viral isolates can be determined using serotype specific monoclonal antibody and/or specific PCR. [145].



Cell culture findings from a vesicle fluid sample taken from a child with HFMD encephalitis. Panel A: control cells; panel B: infected cell lines (Vero cells) showing CPE. EV-A71 was then confirmed by specific PCR. (Pictures courtesy of Prof. D. Q. Ha, OUCRU)

Throat swabs, rectal swabs, vesicle swabs, urine, vesicle fluid and CSF can be used for virus isolation; vesicle and throat swabs provide the highest diagnostic yields with a recovery rate of around 49% and 48%, respectively [146]. Although difficult to collect, material obtained from vesicles should be sterile so the positive predictive value is higher than that of a throat swab, since enteroviruses can exist in the respiratory and gastrointestinal tracts as part of the normal flora.

1.2.6 Clinical assessment and classification of HFMD

Most HFMD cases will recover without specific treatment. However, a small proportion of patients progress to develop severe complications and may rapidly deteriorate to fulminant disease and death. Four disease stages are generally recognized – HFMD/HA, CNS involvement, ANS dysregulation, and cardiorespiratory failure. Based on the potential for progression through these 4 stages a WHO taskforce came together and delivered guidance for HFMD management in 2010 (Figure 1.10) [83]. According to these recommendations patients presenting with HFMD/HA should be assessed for the warnings signs described below:

- High or refractory fever – thought to be associated with a robust immune response or development of ANS dyregulation
- Vomiting, lethargy, agitation or irritability – may indicate onset of CNS involvement
- More definitive neurological signs such as myoclonic jerks, truncal ataxia, nystagmus may also be seen as early signs of CNS involvement.
- Mottled skin and dyspnea/tachypnea – may indicate ANS dysregulation.

Based on these WHO guidelines, the Vietnamese Ministry of Health (MoH) released the following guide for classification and management of HFMD (Table 1.4). Briefly, Grade 1 represents classic HFMD without any complications. Patients with Grade 2 disease show some evidence of central nervous system involvement, usually manifesting as myoclonus. This grade is further split into Grade 2a, in which there is a reported history of myoclonic jerks, and Grade 2b, in which medical or nursing staff observe the myoclonic jerks. In Grade 3 disease there is evidence of ANS dysregulation, while patients with Grade 4 disease have cardio-pulmonary failure [147]

Figure 1. 10: Management algorithm of Hand, Foot and Mouth disease (WHO-2011)

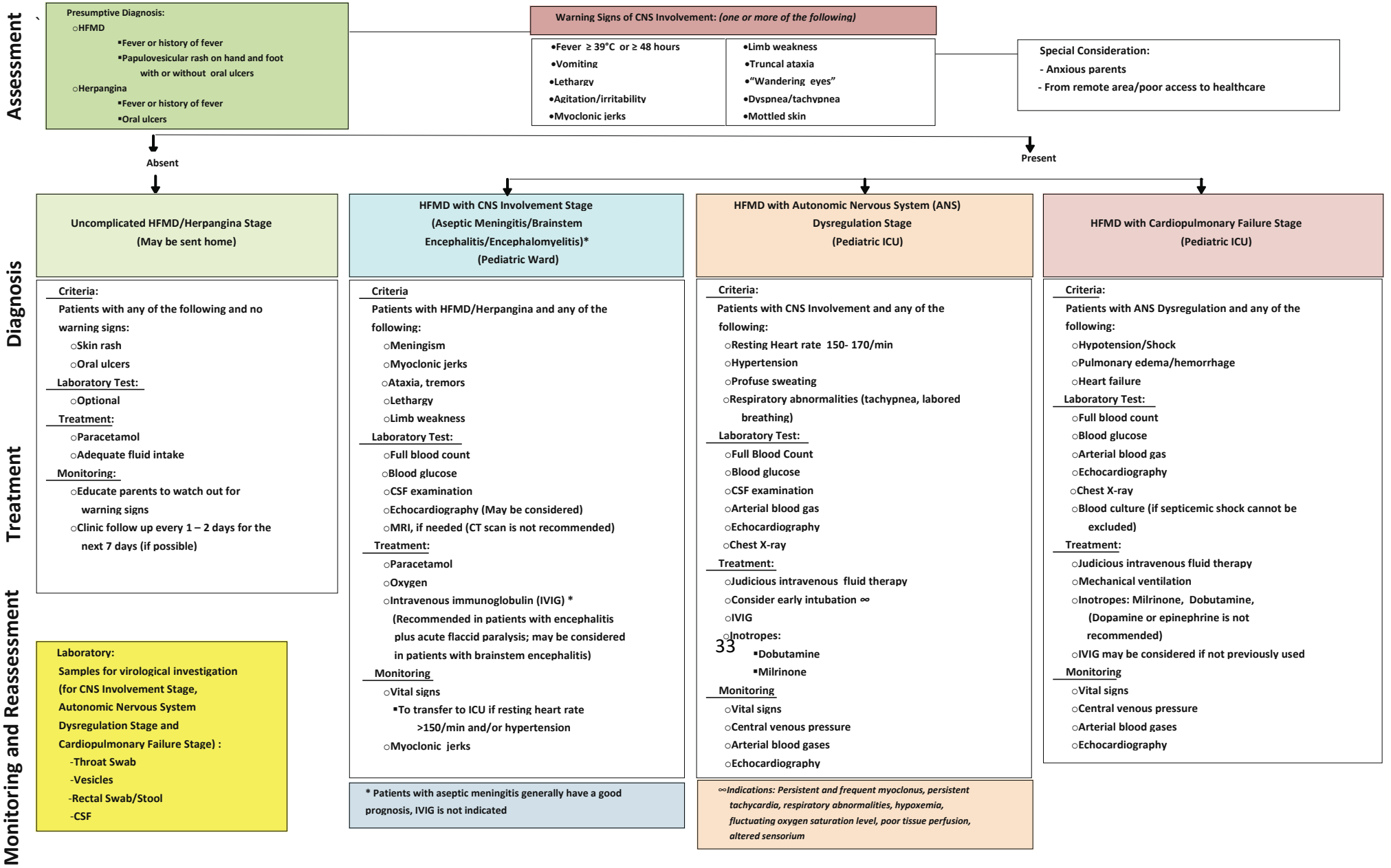


Table 1.4: Vietnamese MoH HFMD Classification and Management Guidelines (March 2012) *

Classification	Signs/symptoms	Suggested Management
Grade 1	Oral ulcers and/or vesicular rash on the hands, feet, and/or the buttocks	OPD care, with advice sheet for family Careful observation for warning signs
Grade 2a	Grade 1 AND Myoclonic jerks observed by the family (not witnessed by medical staff) Legarthy, agitation/irritability Fever ≥ 39°C for ≥ 48 hours. Vomiting	Hospitalization Oral phenobarbitone Vital Signs Monitoring: every 4-6 hours following standard protocols
Grade 2b - Group 1	Grade 1 AND Myoclonic jerks witnessed by medical staff or by the family (≥ 2 jerks/30 minutes or 1 jerk and stupor) Resting pulse rate > 130/min but <150/min (adjusted for fever*)	Admit to HDU/PCIU IV Phenobarbitone Antipyretics Vital Signs Monitoring: every 1-3 hours for minimum 6 hours until stable
Grade 2b - Group2	Grade 1 AND witnessed myoclonic jerks accompanied by one of the following findings: Continuous limb tremor, limb weakness or paralysis, or drowsiness (provided no hypoglycemia) Resting pulse rate > 150 /min (adjusted for fever*) Fever ≥ 39.5^{o}C (rectal) and unresponsive to antipyretics > 4 hours	Give oxygen IV Phenobarbitone Antipyretics Start IVIG – 2g/kg in two divided doses Check: FBC, CRP, blood sugar, and consider lumbar puncture Vital Signs Monitoring: every 1-3 hours for > 6hrs
Grade 3	Serious complications in CNS or cardiopulmonary systems: Pulse >170 /min* Profuse sweating Stage 1 hypertension (SBP > 95th centile for age) Respiratory abnormalities: tachypnea, labored breathing Muscle hypertonia Coma (Glasgow coma score < 10)	Oxygenation Consider need for ventilation IV Phenobarbitone and IVIG Milrinone if stage 2 hypertension (SBP > 99th centile for age + 5 mm Hg) Dobutamine if HR> 170 bpm Consider additional fever control measures Invasive blood pressure monitoring Check: FBC, CRP, blood sugar, and consider lumbar Puncture Vital signs monitoring: every 30-60 mins for ≥ 6 hrs.
Grade 4	Severe complications: Acute pulmonary edema Cardiac collapse SpO2 < 92% with cannula oxygen 6 litres/min) Respiratory arrest or gasping respiration	Intubation and ventilation IV Phenobarbitone Dobutamine; Fluid challenge Antipyretics, Access CVC plus invasive BP monitoring Vital signs monitoring: every 15-30 mins for ≥ 6 hrs.

*: Old version classification (Jun 2011): Grade 2b group 1: HR > 150 /min, Fever ≥ 39.5^{o}C ; Grade 2b group 2: did not include HR and Fever criteria; Grade 3: Stage 2 hypertension (SBP > 99th centile for age + 5 mm Hg). Other criteria did not change.

1.2.7 Management

Most HFMD infections are mild and self-limiting, and do not require specific treatment or hospitalization. However, patients who develop complications, particularly respiratory distress or cardiac failure should be closely observed in an intensive care unit (ICU) or high dependency unit (HDU), since respiratory support and hemodynamic stabilization are important in life support during the severe stage of disease [148].

WHO and Vietnamese MoH guidelines focus on early supportive care with careful observation to recognize disease progression as the cornerstone of case management (Table 1.4). These guidelines advocate use of oral or intravenous (IV) phenobarbital to treat myoclonic jerks and irritability. Warning signs are indications for transfer to HDU for close observation. Intravenous immunoglobulin (IVIG) is recommended for the treatment of Grade 3 and 4 disease, and Grade 2b if the clinician is particularly concerned - however there is no formal evidence to support its use, only some observational studies, indirect evidence and expert opinion [83, 149, 150]. Similarly milrinone, a phosphodiesterase-3 inhibitor, is recommended for the treatment of hypertension on the basis of limited evidence (see below) [117]. Respiratory support is indicated for severe respiratory distress or respiratory failure, and for patients with shock. Hemofiltration has been used in severe cases who do not improve with aggressive intensive care including ventilation, inotropes and heatstroke management [151]. Current Vietnamese MoH guidelines recommend that hemofiltration should be considered as a potential therapeutic option for patients with ANS dysregulation and severe refractory fever. This idea is based on the theoretical potential to remove cytokines from the blood [152-154] rather than in order to reduce the temperature directly by using cool replacement fluids [155].

1.2.7.1 Antiviral therapy

As yet no specific antiviral agents for HFMD have been licensed by the FDA, although some antivirals have demonstrated effects against replication of enterovirus strains in vitro, in mice and in early phase clinical trials. Pleconaril is a broad spectrum antipicornaviral agent that binds to the hydrophobic pocket in the viral capsid, inducing conformational changes which lead to altered receptor binding and viral

uncoating [156]. At a concentration of ≤ 0.18 microM, pleconaril inhibited replication of 90% of the most common enteroviruses isolated in the Unite States in the 1970s and 1980s [157]. In a multicenter, double-blind placebo-controlled study of pleconaril in adult enterovirus meningitis, a modest benefit was demonstrated in reducing the severity and duration of symptoms in a subgroup of patients with more severe disease [158]. However, in a randomized placebo-controlled control trial in infants with enterovirus meningitis, there were no significant differences in the duration of hospitalization or symptoms between the two groups [159]. More recently however, in a randomized control trial involving 61 neonates with enteroviral sepsis, participants who received pleconaril were more likely to survive than the placebo recipients [160]. A number of other antiviral compounds, including vapendavir and pirodavir, have been assessed for their effect on enterviruses in vitro, but the results to date have not been very promising [161, 162].

1.2.7.2 Immunoglobulin

There is no formal evidence of a significant effect in clinical practice, but IVIG was used for patients with agammaglobulinemia and enterovirus meningitis thirty years ago, with variable success [22, 163]. IVIG has also been used to treat viral myocarditis [58, 149]. In the latter report 2 gm/kg IVIG was given to 21 children with acute myocarditis, with improved left ventricular function demonstrated compared to a historical control group that received standard treatment without IVIG.

For severe HFMD there is also no formal evidence of a beneficial effect, although it is recommended by several bodies including WHO and the Vietnamese MoH for severe cases with neurological involvement. There is some indirect evidence to support this recommendation however. High-dose IVIG treatment activates regulatory T cells (Tregs) selectively and enhances their suppressive function in humans in vivo [164], and also results in down-regulation of IL-6 in vitro [165]. Plasma levels of various cytokines including IFN-gamma, IL-6, and IL-8 significantly decreased in HFMD patients with ANS dysregulation or pulmonary edema after administration of IVIG in one case series [166]. Moreover, using human plasma derived IVIG containing neutralizing

antibodies against EV-A71 protected against lethal EV-A71 challenge in a dose and time dependent manner in a suckling mouse model [167].

1.2.7.3 Management of ANS dysregulation

Management of ANS dysregulation focuses primarily on hemodynamic abnormalities, particularly hypertension. Milrinone, a phosphodiesterase-3 inhibitor, was used not only to control high blood pressure but also to support myocardial function in a group of 24 children with severe HFMD compared to historical controls in one study from Taiwan [117]. Milrinone also reduced mortality in patients who developed pulmonary edema associated with HFMD in a small open-label randomized clinical trial in Viet Nam [168]. It is now the recommended therapy for management of severe HFMD with ANS dysregulation, particularly hypertension, in Vietnam. Currently the MoH guidelines assign the threshold indication for use of milrinone at a systolic blood pressure (SBP) over the 99^{th} centile for age plus 5 mm Hg, which approximates to the internationally accepted definition of Stage 2 hypertension in children [169]. Unfortunately however, clinical failures still happen despite high dose milrinone and a number of children go on to require haemofiltration and ventilatory support, the next steps recommended in the MOH guidelines [170].

Considering alternative therapies, magnesium sulfate (MgSO4) had been widely used in controlling the ANS dysregulation seen in severe tetanus. MgSO4 was associated with significantly reduced requirements for drugs to control muscle spasms and cardiovascular instability in a randomized controlled comparison with placebo in adult patients with severe tetanus [171], and use of MgSO4 was associated with reduced circulating and urinary catecholamine concentrations [172, 173]. In vitro, it also has been shown to reduce catecholamine secretion from peripheral nerve endings and the adrenal medulla [174]. MgSO4 has been used in the treatment of eclampsia, severe asthma, pulmonary hypertension, and during anesthesia for spinal cord lesions, to manage ANS associated hypertensive crises [136, 137, 175, 176]. Intrapartum use of $MgSO_4$ in women with preeclampsia was also associated with reduced cytokine levels in the women and their babies [177]. Similarly, use of magnesium in a small number of

patients with aneurysmal subarachnoid haemorrhage was associated with reduced serum levels of certain inflammatory cytokines [178].

MgSO4 was first reported as a potential alternative treatment for severe HFMD cases who presented with hypertension in Hong Kong [179]. Based on this report, and given my experience in using MgSO4 in neonates with severe tetanus, in late 2011 I decided to try this as second line therapy in HFMD cases that did not show a good response to milrinone. In an initial 10 EV-A71 confirmed HFMD cases with ANS dysregulation that I managed at the Hospital for Tropical Diseases (HTD) over a 4 month period $MgSO_4$ was added when hypertension remained poorly controlled despite high dose milrinone (up to 0.75 μg/kg/minute) in all cases the BP reduced within 30-60 minutes and remained stable subsequently on a continuous magnesium infusion for 48-72 hours [180]. No patient required hemofiltration, although 2 of 10 cases were ventilated because of respiratory distress. By comparison, during 2011 before MgSo4 was being used on the Paediatric Intensive Care Unit (PICU) at HTD, of the 35 cases treated with milrinone alone 9 required ventilation (9/35, 26%, unpublished data).

1.2.8 Prevention

Vaccination is the best way to protect individual children from infectious diseases and also limit wider dissemination of the disease. Several EV-A71 vaccines have been trialed recently but as yet none have been approved for deployment in public health programmes.

A phase 1 study assessing a formalin-inactivated EV-A71 vaccine candidate showed production of cross-neutralizing antibodies against EV-A71 subgenotypes B1, B4, B5, and C4A in 60 healthy adults [181]. Two phase III trials have been conducted in children in China recently, involving slightly different EV-A71 vaccines [182, 183]. The safety profiles were acceptable, and in the two-year follow-up to the second trial there was 94.7% efficacy against EV7-A71 associated HFMD Thus these studies indicate that an approved EV-A71 vaccine may be available in near future.

Currently however, no vaccines are approved. Prevention relies on simple and effective public health measures such as frequent hand washing to prevent transmission of enteroviruses. In hospital situations, other measures such as wearing

masks or protective clothing are not recommended, and isolation is only considered necessary in neonatal wards [18, 184].

1.3 Knowledge gaps and objectives of this thesis

Although HFMD was widespread in Vietnam in 2011/2012 and played a major role in childhood morbidity and mortality, there was little published information available describing the spectrum of severe disease, the pattern of autonomic dysregulation, or the clinical risk factors or predictors for poor outcome in these patients. At that time also, the recommended management strategies for severe disease were based primarily on expert opinion and one small study [83, 117, 168]. In our experience in HCMC milrinone, the recommended treatment for ANS dysregulation with hypertension was not always effective, and there were preliminary indications that MgSO4, a cheap, easily available and apparently safe drug, might be effective. However, there was no formal evidence to support use of MgSO4 in this way for severe HFMD cases.

My overall aim in conducting this research programme was to fill in some of these knowledge gaps, with the following main objectives:

a) To systematically describe the clinical features of all HFMD cases seen in PICU in one referral centre in Vietnam in 2011-2012, together with current management strategies and disease outcomes, and to investigate relationships with the enterovirus serotypes in circulation during this period.
b) To investigate possible risk factors, identified during the first 24 hour in PICU, for subsequent progression to one of a number of well-defined severe outcomes.
c) To evaluate the effect of MgSO4 on control of hypertension and other manifestations of autonomic dysfunction, in children with HFMD.
d) To describe the safety profile of MgSO4 when used in children with severe HFMD and hypertension.
e) To describe catecholamine levels as biomarkers of sympathetic activity in severe HFMD cases, and assess the impact of MgSO4 on these levels.

By addressing these aims, I hoped to provide clinicians with a detailed clinical picture of severe HFMD and the risk factors for disease progression, so they can respond effectively when major outbreaks happen. Secondly gaining knowledge about the efficacy and safety of MgSO4 in managing severe HFMD, would be useful whether the results were positive or not. Clearly if a positive effect could be demonstrated this would be of value for future management recommendations, but it is just as important to know if there is no benefit to use of MgSO4 before it becomes an established therapy.

Chapter 2

MATERIALS AND METHODS

In this chapter I will describe the general clinical, laboratory, and statistical methods used for the research presented in Chapters 3 – 5 of this thesis. Specific methods used for particular studies will be presented in the relevant chapters.

2.1 Clinical studies

All the clinical activities for the studies described here took place on the Pediatric Intensive care Unit (PICU) at the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City. At HTD teenagers aged over 15 years are usually admitted to adult wards, so the studies described only involved children below 15 years of age. At the time of most of this work, HTD was admitting around 2,000 - 4,000 patients with suspected HFMD each year, most of whom were managed on the pediatric infection wards, but around 15% were admitted to PICU because of severe disease.

Initially I had planned to carry out the randomized control trial described in Chapter 4 on two sites, HTD and Children's Hospital 1 (CH1). At CH1 between 4,000-6,000 children with the same severity of HFMD were seen each year and usually managed at the Infectious Disease Department. This is a very large department (90 beds) that includes a high dependency unit (HDU, 10 beds) where severe patients were managed except for very serious cases who needed highly specialized interventions such as hemofiltration, who were transferred to the main PICU at CH1.

2.2 Ethics

All studies were carried out in accordance with international standards for the ethical conduct of research involving human subjects.

Ethical approval for the retrospective file review (Chapter 3) was obtained from the Scientific and Ethical Committee of HTD. Since the study was retrospective and anonymous, informed consent was not obtained from the parent/guardian of the individuals involved. All substantial amendments to the original approved documents,

including requests for further serotyping and sequencing to identify the serotype of enterovirus and subgenogroups of EV-A71, and also to collect the additional information on hemodynamic parameters (see Chapter 5) were also approved by the HTD Committee.

The randomized controlled trial described in Chapter 4 was conducted in compliance with the current revision of the Declaration of Helsinki (Seoul 2008) and the terms of approval of all supervising Ethical Committees. The study protocol and its associated documents were reviewed and approved by the following committees: The Vietnam MoH's Evaluation Committee on Ethics in Biomedical Research: The Scientific and Ethical Committee of HTD, The Science and Ethics Committee in Biomedical Research of CH1, and the Oxford Tropical Research Ethics Committee (OxTREC, University of Oxford). The study was managed by the OUCRU Clinical Trials Unit (CTU) in compliance with the ICH Guidelines of Good Clinical Practice, the Vietnamese MoH Guidelines of Good Clinical Practice and the relevant institutional and regulatory requirements. A parent/guardian gave written informed consent before enrolment for all participants enrolled in the RCT. Further details on this process are described in Chapter 4.

2.3 Laboratory methods

2.3.1 Standard laboratory tests

Hematology: A full blood count was performed at least once, usually during the first 24 hours after PICU admission, and then subsequently depending on the assessment of the attending doctors. Tests were performed in the routine hematology laboratory of HTD using the ADVIA 2010i haematology analyzer (Siemens Healthcare, Germany).

Biochemistry: Blood sugar and CRP, using the routine HTD laboratories, were tested routinely at admission. CK-MB and Troponin I were measured in the routine labs whenever patients had signs or symptoms of cardiovascular compromise, using the Cobas C501 (Roche Diagnostics GmbH, Mannheim, Germany) and Archtect 4000SR (Abbott) analyzers.

ELISA for catecholamine concentration: In the randomized trial, urine and plasma catecholamine concentrations were measured using the BI-CAT®Adrenaline and

Noradrenaline ELISA Assay Kit (EAGLE BIOSCIENCES, www.eaglebio.com), following the manufacturer's instructions. The catecholamines were first extracted out of the sample, then derivatized for antibody recognition and to enhance their stability in the solid phase, and thereafter detected using antibodies specific for catecholamines. The amount of antibody bound to the solid phase catecholamine is inversely proportional to the catecholamine concentration of the sample.

2.3.2 Real-time PCR for detection of enteroviruses

Most, but not all, of the molecular biology diagnostics were performed in the OUCRU laboratory by staff from the Emerging Viral Infection research group, although some PCR testing was done in the routine HTD laboratory. Both labs use the same standard protocol that was developed and published by OUCRU laboratory [185].

The process of specimen collection and storage: Diagnostic material from the throat, nose, vesicle fluid or other specimens was collected on appropriate swabs that were placed immediately in viral transport medium and brought to the laboratory. The material was divided into three aliquots and stored at -80^{0}C prior to analysis.

Nucleic acid extraction: Viral RNA was extracted from 140 µl clinical specimens (throat, rectal or vesicle swabs, or CSF) together with 20 µl of Equine Arteritis Virus (EAV) functioning as an internal control, using QIAamp Viral RNA MINI Kits (Quiagen, Hilden, Germany). The process of extraction was performed following the instructions from the manufacturer. Viral RNA was then washed out in 50 µl elution buffer and stored at -80^{o}C until used.

Internally controlled one-step multiplex real-time RT-PCR: From Jan 2011 to August 2011, Taqman Probes (enterovirus and EAV probes) supplied by Proligo® Reagents were used for detecting enteroviruses in the OUCRU molecular laboratory. After that period, one-step multiplex real-time RT-PCR was employed to simultaneously detect EVs and EV-A71 using the SuperScript III One-Step gRT-PCR System with Plantinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) and was performed in a LightCycler 480 II machine (Roche Diagnostics GmbH, Mannheim, Germany).

Primer and probe sequences that were adapted from previous studies of Scheltinga et al., 2005, Beld et al., 2004 and Khanh et al [186-188] were optimized and validated at the OUCRU laboratory (Table 2.1) [185].

Table 2.1: Primer and probe sequences and concentrations used in one-step real-time RT-PCR reaction

Name	Concentration (µM)	Sequence (5' – 3')	Note
EAVF	10	CAT CTC TTG CTT TGC TCC TTA G	Internal control [188]
primer	10	AGC CGC ACC TTC ACA TTG	
EAVR	2.5	FAM-5'-	
primer		CGCTGTCAGAACAACATTATTGCCCAC-	
EAV-probe		3'-BHQ1	
ENT-F	10	5'-CCCTGAATGCGGCTAAT-3'	Enterovirus specific primers and probe [186]
ENT-R	10	5'- ATTGTCACCATAAGCAGCC-3'	
ENTr-probe	5	Cy5-ACCCAAAGTAGTCGGTTCCG - BHQ3	
EV-A71-634F	10	GGAGAACACAARCARGAGAAAGA	EV-A71 specific primers and probe [187]
EV-A71-743R	10	ACYAAAGGGTACTTGGAYTTVGA	
EV-A71-probe	1	Cyan500-TGATGGGCACDTTCTCRGTGCG-BHQ1	

Note: FAM = Carboxylfluorescein; Cy5 = Cyanine 5; Cyan500= Cyan 500 NHS ester; BHQ= black hole quencher; R= A or G; Y= T or C; V=A, C or G; D=A, G or T.

5 µl of extracted viral RNA was mixed with 12.5 µl 2X RT-PCR Reaction Mix (Invitrogen), 1 µl primers and probes of EAV, EVs and EV-A71 respectively (Table 2.1), 0.5µl of enzyme mix (Invitrogen). The real-time RT-PCR reaction was carried out in that mixed solution. One cycle of reverse transcription and polymerase activation were performed under thermocycling conditions that consisted of 60^{o}C for 3 minutes, 53^{o}C for 15

minutes and 95°C for 2 minutes. The next step of 45 cycles of PCR amplification was done with conditions consisting of 95°C for 15 second, 53°C for 1 minute and 72°C for 15 second.

Principles for interpreting the results: The final result was identified as positive if a) positive controls showed Crossing point (Cp) values of 30+/-1; b) negative controls were negative, and c) the tested sample showed a positive result with Cp value equal to or less than 40. The final result was considered as negative if a) negative controls were negative; b) positive controls showed Cp values of 30+/-1; c) IC showed positive result with Cp value of between 30 and 33; and d) the tested sample was negative. A sample was considered as inconclusive if a) negative controls were positive; b) and/or the IC result was out of the expected range; and c) amplified signal was not observed for EVs/EV-A71.

2.3.3 Nested PCR

The remainder of the extracted RNA of samples that were EV-PCR positive but EV-A71 PCR negative were stored at -80^{0}C. Later those stored specimens were used to identify the enterovirus serotypes using a nested PCR targeting the VP1 gene sequence (Figure 2.1). Primer sequences were adjusted from W.A. Nix et al., 2006 [189] (Table 2.2).

The first round of reverse transcription and amplification was performed by using SuperScript III One-step RT-PCR with five micro liters of the extracted viral RNA and a total reaction volume of 20 µl containing 10 µl of 2x reaction buffer (provided with the kit), 0.5 µl of SuperScriptIII RT/Platinum Taq High Fidelity mix (Invitrogen), 1 µl of first-round primers mix Plantinum Taq High Fidelity and outer primers SO24 and SO22 (Table 2.2). One cycle of reverse transcription under the thermocycling conditions of 55°C for 3 minutes, 22°C for 10 minutes and 42°C for 30 minutes, followed by one cycle at 95°C for 1 minute and then 40 cycles of amplification consisting of 94°C for 30 seconds, 42°C for 30 seconds and 60°C for 1 minute, and a final extension step at 72°C for 7 minutes.

The second round amplification was done in a final reaction volume of 25 µl containing 1 µl of the first round RT-PCR product, 23 µl mix of Platinum PCR Super (Invitrogen), and 0.5 µl of each of the AN89 and AN88 primers (Table 2.2). The thermocycling

conditions for the first cycle were 95°C for 1 minute, followed by 40 cycles at 94°C for 20 seconds, 60°C for 30 seconds, and 72°C for 20 second, and final extension at 72°C for 7 minutes.

Amplified products of the second round PCR were then separated on 1% agarose stained with Nancy-520, and observed under UV light. The expected size of the amplified product is between 350 – 400 base pair.

2.3.4 Sequencing, sequence analysis and determination of EV serotype and EV-A71 subgenogroups

Primers AN232 and AN233 were used for identifying EV serotypes while EV-A71-VP1-3F and EV-A71-VP1-703R were used for identifying the subgenogroups of EV-A71. Sequencing of VP1 PCR products of the expected size was performed in both directions using those primers and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) in an ABI 3130XL DNA sequencer (Applied Biosystems) (Table 2.2).

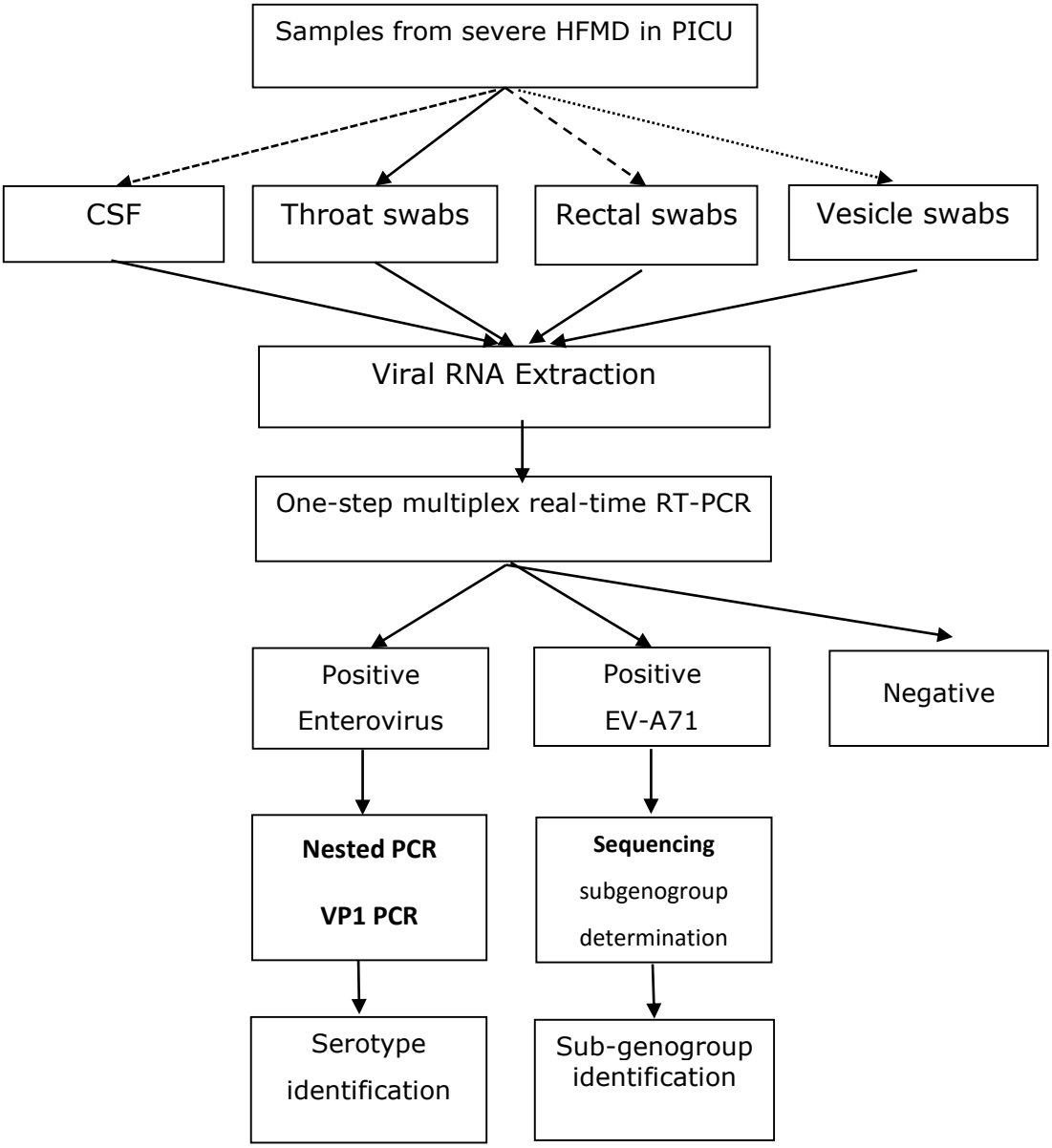
5 µl of diluted purified PCR product, 4 µl of BigDye (Applied Biosystems), 2 µl of buffer, 8 µl of molecular biological water, and 1 µl primer (AN232 or AN233) were mixed for the sequencing reaction. This reaction was performed at 95°C for 1 minute, followed by 30 cycles consisting of 94°C for 20 seconds and 60°C for 2 minutes.

Obtained sequences were manually assembled and edited using Contig Express– a component of Vector NTI Suit 7 (Informax Inc., NY, USA). EV serotypes and EV-A71 subgenogroups were determined by employing an online EV typing tool (http://www.rivm.nl/mpf/enterovirus/typingtool/) as described by Knoneman, A. et al., 2011.

Table 2.2: Primers sequences & concentrations of nested RT-PCR & sequencing reactions

Name	Sequence (5'→3')	Note	Working concentration (µM)
SO224	GCIATGYTIGGIACICAYRT	1st round RT-PCR	10µM of each **(Primer mix)**
SO222	CICCIGGIGGIAYRWACAT		
AN32	GTYTGCCA		
AN33	GAYTGCCA		
AN34	CCRTCRTA		
AN35	RCTYTGCCA		
AN89 (forward)	CCAGCACTGACAGCAGYNGARAYNGG	2nd round PCR	10
AN88 (reverse)	TACTGGACCACCTGGNGGNAYRWACAT		
AN232	CCAGCACTGACAGCA	Serotype sequencing	10
AN233	TACTGGACCACCTGG		
EV-A71-VP1-3F	AGAYAGGGTGGCRGATGT	EV-A71 Sequencing	10
EV-A71-VP1-703R	CTGAGAACGTGCCCATCA		

Figure 2.1: Algorithm for enterovirus serotyping and sequencing of genogroups of EV-A71



2.4 Data management

2.4.1 Data entry, checking and cleaning

Together with a team of experienced study doctors I checked the information in the paper CRFs used for the file review after the data had been extracted from the hospital notes and before sending them to the data entry team at CTU.

The clinical and laboratory data of all studies were stored on an ICH-GCP compliant clinical data management platform called "CLIRES" that was developed at OUCRU. For the trial and cohort study, all data were single entered by study nurses, then checked again by the study coordinator and data entry team members, while for the descriptive datasets a double data entry method was used with inbuilt logical checks. All queries generated by the logical checks were referred back to me for cross-checking and correction based on the original source documents.

2.4.2 Missing data

A data assessment analysis was performed to check for duplicate records of the same patient and for missing data. Specific types of missing data were identified. Random missing data were accepted as truly missing and imputed if necessary for analyses using multivariable logistic regression (see Chapter 3). Non-random missing data were cross-checked with the hospital notes and the hospital's electronic database, and the CLIRES system was updated with the new information.

2.5 Statistical methods

I used R studio software version 1.0.143 (https://download1.rstudio.org/RStudio-1.0.143.dmg) to analyze the data and draw the figures in this thesis.

Unless otherwise specified summary statistics are absolute count and percentage for categorical variables and median (interquartile range-IQR) for continuous data. However, for data involving small patient numbers median (range) is used. In the descriptive study, odds ratios (OR) and p-values for categorical variables were based on logistic regression models. The mean difference and p-value for continuous data were based on linear regression models. For variables with multiple categories,

ordered logistic regression was applied to calculate OR and significance tests. Multivariable logistic regression was used for prognostic factor analysis with stepwise backward model selection. Pearson correlation was used to evaluate relationships between continuous variables.

In the MgSO4 trial, log-binomial regression, linear regression, and Cox regression were used for binary outcomes, continuous outcomes, and time-to-event outcomes, respectively. Measures of association are displayed as relative risk (RR), mean difference, and hazards ratio, depending on the type of outcome.

P-values < 0.05 were considered statistically significant and 95 % confidence intervals (CI) were applied for all tests.

Additional details on the specific statistical methods used will be described in the relevant chapters.

Chapter 3

OVERVIEW OF SEVERE HFMD: CLINICAL FEATURES AND RISK FACTORS

3.1 Introduction

The clinical features of HFMD have been described in some research studies conducted during different outbreaks, but it is rare that the reports focus on severe cases or describe the evolution of the clinical features systematically over time [104, 190]. In 2011, WHO included a clinical description in their HFMD guidelines [83], based largely on retrospective reports describing relatively small patient numbers and mostly focused on EV-A71 infection [74, 125, 191]. However, one prospective study described the clinical features and detailed virology in almost 800 children, of whom 277 had EV-A71 infection [192]. Since 2011, this guideline has been adopted in many countries where HFMD has become a problem, including Vietnam. Some studies that focused on the severe end of the spectrum mostly came from China, and although these studies described the clinical features of severe cases in large patient populations, many were published in the Chinese language making them inaccessible to the general reader [193-195], or used alternative less specific classification systems for severe disease to the conventional WHO system [196, 197].

The 2011 outbreak of HFMD in Viet Nam continued to have a major impact on Vietnamese children during 2012, but mortality was reduced in comparison to 2011. Considering all reported cases throughout Vietnam the mortality rate was 170/113,113 (0.15 %) in 2011 compared to 45/157,654 (0.03 %) in 2012 [198]. The Vietnamese MoH continued to revise the strategy for diagnosis and treatment of children with HFMD, issuing two revisions, the first in June 2011, the second in Mar 2012 with minor change (described in chapter 1), to the guidelines during this period, and this may have resulted in improved outcomes. However, alterations in the predominant serotypes circulating may also have occurred over time, potentially due to changing population immunity after a period of exposure to one serotype [199-201].

Moreover, identification of risk factors that predict the likelihood of progression to

severe outcome is very important for clinicians, especially during outbreak situations. A number of different features, including age, sex, place of living, illness day at presentation, fever, heart rate, abnormal respiratory pattern, pleocytosis, and hyperglycaemia have been considered as risk factors/prognostic factors for severe disease (Table 3.1) [112, 194, 202-209], but in general these reports described associations between the factors with the outcome, rather than identifying features that occurred before the development of severe disease. In one prospective study peak temperature ≥ 38.5°C, history of lethargy, total duration of fever for more than 3 days were predictors of neurological complications [209], but longitudinal relationships between these factors and outcome were not clearly defined.

In this chapter I will systematically describe the spectrum of disease, management strategies and outcomes, for all patients admitted to PICU from January 2011 to December 2012 aiming to expand knowledge about HFMD in general and also investigate associations between enterovirus serotypes and clinical presentation. In addition I will present some analyses looking at risk prediction for subsequent deterioration, assessing the ability of features observed within the first 24 hours in PICU to predict severe outcome. These observations could be important for development of intervention strategies aiming to improve clinical management and achieve better outcomes in the future.

Table 3.1: Overview of studies assessing risk factors for severe HFMD from 2012 to 2016 [112, 194, 202-209]

Reference	Year	Country	Outcome definition	Study design	No of Patients	No of severe cases	Risk factors
							Factors
Ooi M.H. et al	2009	Malaysia	Neurological complications	Prospective	725	102	Peak temperature ≥ 38.5°C, History of lethargy, total duration of fever ≥ 3 only
Kim S. J. et al	2013	Korea	Neurological complications	Retrospective	168	88	Headache, neurologic signs
Wang Q. et al	2014	China	Any signs and symptoms of respiratory and circulatory disturbance involved the CNS	Case- control	120	60	≥ 38.5°C, Rash type, Lethargy
Li W. et al	2014	China	Brainstem encephalitis, and/or pulmonary edema	Retrospective	1500	350	RR>26/min, Age<4 yrs, Glycemia>8.3 mmol/L, Lymphocyte>40%, and ALT>40 U/L
Fang Y. et al	2014	China	Neurological, respiratory, or circulatory complications or death.	Meta-analysis	*	*	Fever duration ≥3 days, ≥ 37.5 ° C, lethargy, hyperglycemia, vomiting, increased neutrophil count, EV-A71 infection, and young age
Chew S. P.. et al	2015	Singa-pore	Death, encephalitis, meningitis, myocarditis, pulmonary edema or acute flaccid paralysis	Case-control	72	24	Evidence of hypoperfusion, seizure, altered mentation, meningeal irritation, tachycardia, tachypnea, increased neutrophil count and EV-A71 positivity
Liu R.H. et al	2015	China	Requirement of mechanical ventilation	Retrospective	63	63	Poor peripheral circulation, pulmonary edema, white blood cell counts, blood lactate, glycemia
Song C. et al	2015	China	Cardiopulmonary collapse	Retrospective	176	31	Vomiting, circulatory disturbance, EV-A71 infection, dysfunction of respiratory rhythm and high level of brain natriuretic peptide
Owatanapanich S. et al	2016	Thailand	Severe enterovirus infection	Retrospective	156	25	Absence of oral lesions, seizures, and drowsiness/lethargy
Long L. et al	2016	China	Cardiopulmonary failure	Retrospective	1,125	976	Young age, fever duration ≥3 days, coma, limb weakness, drowsiness and ANS involvement

*: The number of patients included from the total of 19 studies varied depending on the factors assessed in the meta-analysi

3.2 Materials and methods

Clinical features: For this retrospective review of children admitted to PICU with a clinical diagnosis of HFMD from January 2011 to December 2012 I designed a CRF to collect data from the hospital files. File numbers were first identified from the hospital's electronic database in which the final clinical diagnosis and the final overall severity grade were entered by nursing staff at the time of discharge. Files of all patients with a discharge diagnosis of HFMD Grade 2b and above from 2011-2012 were retrieved from the medical records department. However, I was particularly interested in the evolution of signs and symptoms over the first 7 days of illness so patients who were admitted to PICU after day 7 of illness were not included in this analysis.

I trained and supervised two study nurses and several experienced ICU ward doctors to systematically review the files and record the following information: demographic and epidemiological data; history and examination findings at PICU admission; a summary of all clinical events occurring each day with a particular focus on neurological, autonomic and cardiovascular manifestations; details on all interventions during the hospital stay; and information on important clinical outcomes. The CRF was structured to gather data from the daily morning ward round around 8 am, and include a summary of all events in the previous 24 hours. Data collection was helped by the fact that in response to the outbreak the Vietnamese MoH issued guidelines in 2011 indicating that all important signs/symptoms should be documented in patients with HFMD, with a checklist provided for doctors and a nursing chart for nurses to record the essential information (Appendix, page 85) [147]. Such data were usually documented in the hospital files for the time period spent on PICU, but not always for the time prior to admission if managed on other wards, or later when the child had stabilized and was transferred out. Some vital signs, including blood pressure, were not always recorded, especially in young children if they were distressed. Information on the patient's identity (hospital number) was included on the paper CRF for checking purposes, but the data were anonymized at the time of entry to the electronic database.

To gain a general perspective of HFMD at the hospital during the same time-period, I also collected basic information from the main HTD hospital database, for all children discharged with this diagnosis in 2011 and 2012. These data included the age, gender, date of admission, and final discharge classification, all in electronic format.

Additional general information relating to ethics, data extraction and data management, etc. are described in Chapter 2. Definitions for clinical signs and symptoms, complications, disease severity measures/outcomes are shown in Appendix (page 86).

Laboratory investigations: During the 2-year period that we focused on, there were some differences in the types of laboratory data available, because of gradual evolution of the Vietnamese HFMD management guidelines. In the early stages of the 2011 outbreak, the MoH recommendation was to take throat, rectal, and vesicle swabs for laboratory confirmation of enterovirus infection by real-time PCR in all suspected HFMD patients admitted to PICU. Moreover, specific PCR for EV-A71 was only introduced in September 2011, when the laboratory developed the capacity to perform this analysis, but it was only done in cases in which the initial PCR for enterovirus proved to be positive. Later, specimens from cases that were positive with enterovirus but negative with EV-A71 were stored properly at – 80^{o}C, so that when PCR for other enteroviruses had been developed it was possible to go back to the original samples and do further laboratory testing.

Initially lumbar puncture was also recommended in patients who had signs and symptoms of CNS involvement, with CSF to be sent to the hospital laboratory for cell count, biochemical tests, culture and real-time PCR for enterovirus. In addition, WBC, blood sugar, and CRP were to be checked every day for the duration of the PICU stay. However subsequently these guidelines were rationalized due to the extremely heavy workload in 2011-2012 and after preliminary assessment had shown that lumbar puncture, daily WBC, biochemical tests, and real-time PCR in all samples did not help much for diagnosis or management. By 2012 the routine practice for all suspected HFMD cases on admission to PICU had become a) to send a throat swab for enterovirus PCR and b) to request an initial WBC and CRP. Lumbar puncture and repeat

hematology and biochemistry were left to the discretion of the treating clinicians, to be performed if they felt that any of these tests would be contributed to management.

Details for the specific protocols for enterovirus diagnostics are presented in Chapter 2.

Statistical methods: general statistical methodology is presented in Chapter 2. For the analysis to identify risk factors for progression to severe disease, the population of interest was defined as all cases admitted to PICU within the first 7 days of illness with clinically diagnosed HFMD of Grade 2b or above, provided they did not develop any of the predefined outcomes within the first 24 hours after admission. These outcomes included death or occurrence of severe complications that mandate specific therapy according to the Vietnamese M0H guidelines: hemodynamic instability requiring fluid resuscitation or inotropes; respiratory distress requiring ventilation; hypertension requiring milrinone; or high refractory fever requiring hemofiltration.

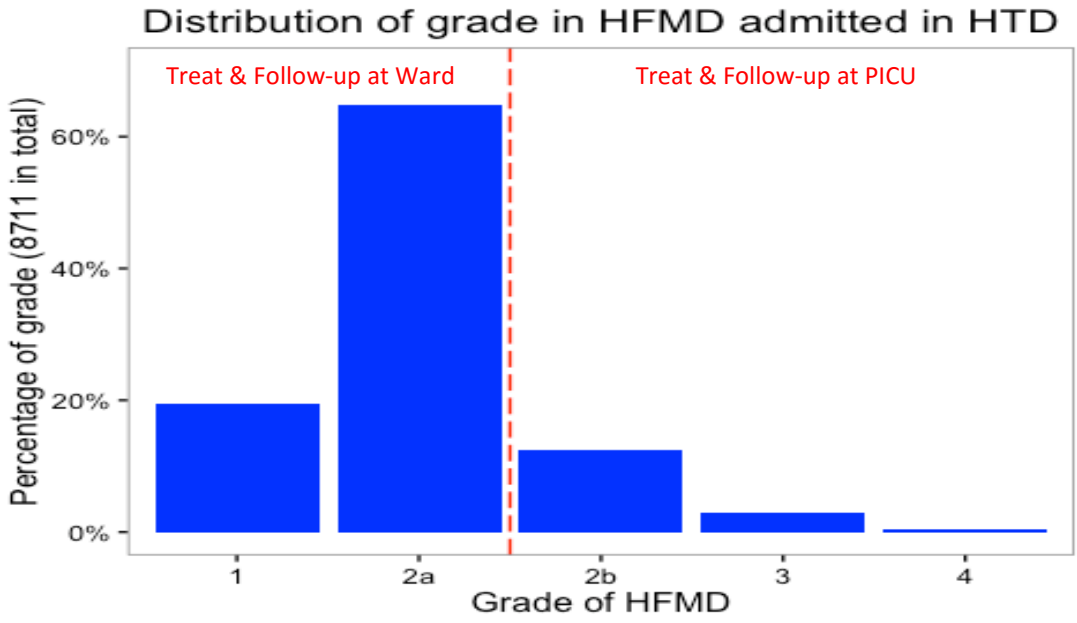
Candidate predictors, all assessed within the first 24 hours in PICU, were chosen based on clinical experience and evidence from the published literature [112, 205, 206]. Signs or symptoms that occurred very infrequently, or that were closely related to each other or the outcomes of interest were not selected. The final list of variables chosen are described in the Appendix, page 90.

Multivariable logistic regression was used for the analysis, with stepwise backward model selection. I carried out the regression once using the available dataset, and a second time after imputation for missing data. To do this I imputed missing values as “yes” if this was the dominant (>50%) response for that variable, and vice versa if the dominant response was “no”. P-values < 0.05 were considered significant and 95 % confidence intervals were applied for all tests.

3.3 Results

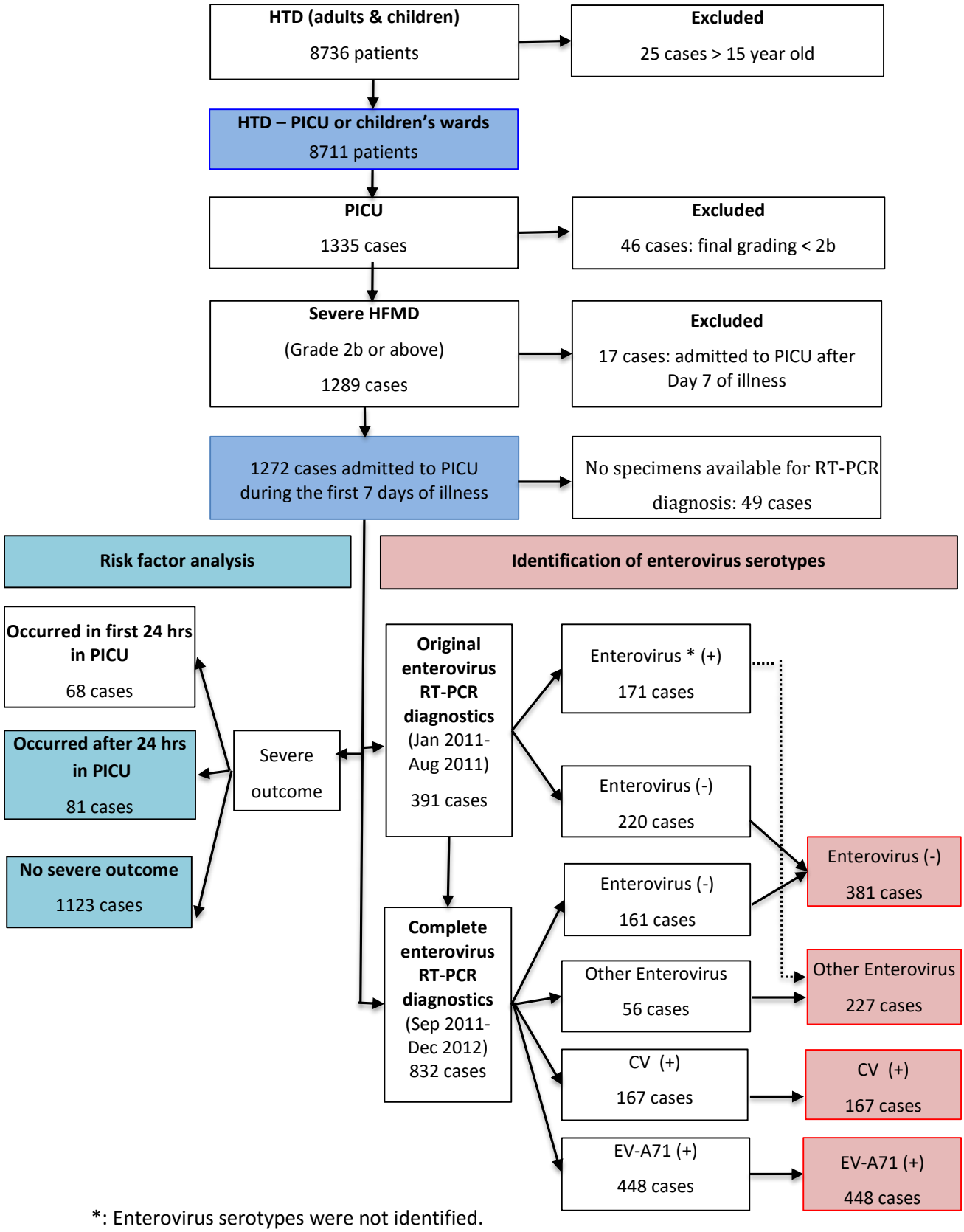
From January 2011 to December 2012, 8736 patients who were admitted to HTD were given a final clinical diagnosis of HFMD at discharge, of whom 8711 children were under 15 years old. Among them, 1335 cases were either admitted directly to PICU, or transferred there from other pediatric departments because of concerns regarding potential CNS involvement or other severe manifestations related to the respiratory or cardiovascular systems. Within this group, 1289/8711 (15%) cases, including 971/8711 (11.1%) Grade 2b, 296/8711 (3.4%) Grade 3, and 22/8711 (0.3%) Grade 4, were classified finally as presenting signs or symptoms of Grade 2b disease or above. The majority, 7422/8711 (85%) cases, presented mild disease only and were classified as Grade 1 (1837/8711, 21%) or 2a (5585/8711, 64%). (Figure 3.1, Figure 3.2).

Figure 3.1: Distribution of severity grades in HFMD patients admitted to HTD during 2011-2012



However, only 1272 of the cases admitted to PICU were admitted there during the first 7 days of illness. In these patients, specimens were not available in 49 cases, leaving 1223 patients for the main analysis. 1206 throat swabs, 60 rectal swabs, 49 CSFs, and 5 vesicle fluid swabs were collected and had RT-PCR performed, although the schedule of tests performed differed according to the time during the study period (i.e. admission before or after September 2011).

Figure 3.2 : Flow diagram showing patient selection for clinical overview (blue boxes) & risk factor analysis (green boxes), and perfomance of diagnostic tests (pink boxes)

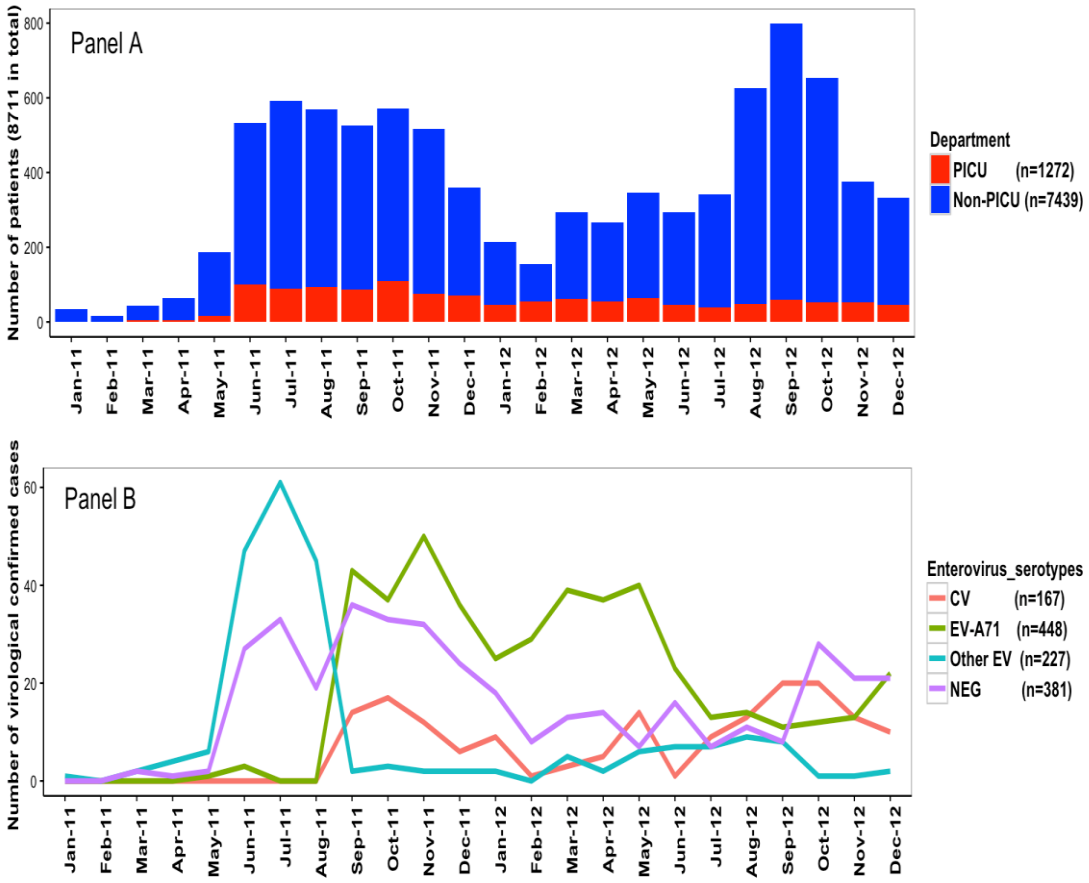


*: Enterovirus serotypes were not identified.

An enterovirus was identified in 68%, 75%, 8%, and 80% of those specimen groups, respectively. Overall, RT-PCR for enterovirus was positive in 842/1272 (66 %) cases. EV-A71 was confirmed in 448/1272 cases (35%), while CV, including serotypes A2, A 4-6, A8-10, A 12, A 16, B4, were found in 167/1272 (13%) cases. In particular, CV-A6 and CV-A10 in CV group were dominant, identified in 73/167 (44%) and 35/167 (21%) cases. Echovirus, including serotypes 6, 9, and 33, were found in 56/1272 (4%) cases; data for these cases were grouped together with the data from children in whom the original group-specific RT-PCR was positive but no specific serotype was identified.

In Figure 3.3 the distribution of HTD and PICU admissions by month is shown for the 2-year period (Panel A). During the early part of 2011 overall numbers were low but from May to December there was a marked rise, followed again by a similar pattern in 2012.

Figure 3.3: Seasonal distribution of HFMD cases in PICU and HTD in 2011-2012 (Panel A), and the enterovirus serotypes (Panel B) identified in the PICU population



This time period corresponds to the rainy season in Viet Nam. Interestingly the PICU admissions did rise correspondingly with the HTD peak in 2011, but then maintained at a relatively constant level per month during 2012 despite a large peak in HTD admissions from August to October 2012. Panel B shows the changing pattern of serotypes identified in the PICU patient group during the same time period. Prior to September 2011 very few cases had a specific serotype identified due to the limited diagnostics available at that time. After September 2011 EV-A71 was the predominant serotype for about 1 year, while CV and Echovirus were mainly found in 2012 (126/176, 72% of all isolates) compared to 2011 (50/176, 28% of all isolates). From the epidemic profile it is possible that the large peak of patients in mid 2011 for whom the enterovirus PCR was positive, but no serotyping was done, may have had EV-A71 infection

3.3.1 Demographic information and clinical features within the first 24 hours of PICU admission

The median (IQR) age of 8377 HFMD cases managed at HTD during the selected time period was 18 (12, 20) months; 7325/8377 (87 %) were 3 years old or younger, and 5204/8711 (60%) were male. The median age in the PICU group, 18 (12, 27) months, was similar to the overall HTD patient group, 1103/1266 (87%) cases were aged 3 years or less and 781/1272 (61%) were male. (Note that in some children in both groups the age was only recorded in years rather than months, reducing the denominator slightly.)

Data for the 1223 cases with a final Grade of 2b or above who were managed on PICU and had viral diagnostics performed are described in Table 3.2, presented according to the virology results. Since the CRF was structured to gather data from the daily morning ward round, and include a summary of all events in the previous 24 hours, the data represent events occurring within varying time intervals since each patient was admitted at a different time. Data was missing for less than 5% of children for most variables. All statistical comparisons presented are between the EV-A71 group and the CV group, using linear or logistic regression models as appropriate.

Table 3.2: Demographic information and clinical features occurring within the first 24 hours, among 1223 children admitted to PICU with a clincial diagnosis of HFMD, presented according to viral serotype

	EV-A71 (N=448)	CV (N=167)	Estimated effect * (95%CI)	Other EV (N=227)	NEG (N=381)
Demographic information					
Sex (F)	168 (38)	63 (38)	1.01 (0.70, 1.45)	94 (41)	146 (38)
Age (months)	21 (13, 30)	16 (10, 24)	5.23 (2.49, 7.97)†	18 (12, 27)	17 (11, 25)
Weight (kg)	11 (10, 14)	11 (9, 13)	0.85 (0.08, 1.61)&	11 (10, 13)	10 (9, 12)
Urban address	232 (52)	103 (62)	0.67 (0.46, 0.96)&	138 (61)	234 (61)
Year of presentation (2011)	180 (40)	49 (29)	1.62 (1.11, 2.39) &	175 (77)	211 (55)
Illness day at PICU admission	3 (2, 4)	2 (2, 3)	0.80 (0.62, 0.98)†	3 (2, 4)	3 (2, 4)
Previous HFMD infection	39 (9)	22 (13)	0.62 (0.36, 1.10))	19 (9)	58 (16)
General signs and symptoms					
High fever (>40^{0}C)	58 (13)	20 (12)	1.08 (0.64, 1.90)	33 (15)	68 (18)
Diarrhoea	7 (2)	2 (1)	1.29 (0.31, 8.69)	5 (2)	17 (4)
Vomiting	10 (2)	5 (3)	0.72 (0.25, 2.36)	13 (6)	15 (4)
Skin lesions and mouth ulcers	277 (63)	93 (58)	1.25 (0.86, 1.80)	123 (58)	126 (35)
Skin lesions only	118 (27)	11 (7)	5.00 (2.73, 10.09) †	58 (27)	82 (22)
Mouth ulcers only	42 (9)	54 (33)	0.21 (0.13, 0.33) †	27 (12)	110 (30)
Target organ involvement					
Myoclonic jerks**	163 (37)	34 (21)	2.17 (1.43, 3.35) †	115 (52)	138 (37)
Cerebellar signs**	11 (3)	5 (3)	0.80 (0.29, 2.58)	25 (11)	16 (4)
Tachypnea for age**	99 (22)	7 (4)	6.45 (3.14, 15.59) †	47 (22)	51 (14)
Irregular breathing**	83 (19)	3 (2)	12.36 (4.54, 50.91) †	24 (11)	22 (6)
Tachycardia (HR>150 bpm)**	262 (59)	100 (60)	0.94 (0.66, 1.36)	135 (61)	212 (56)
High SBP (mm Hg)**	96 (23)	17 (11)	2.35 (1.39, 4.21)&	31 (17)	48 (14)
Skin ANS features **	6 (1)	4 (2)	0.55 (0.15, 2.16)	8 (4)	15 (4)

Missing data in less than 5% of all parameters presented, apart from blood pressure (6.5%), which could not be measured in some young children when distressed/crying. Percentages are calculated on the true denominator for each variable

Summary statistic is absolute count (%) for categorical variables and median (IQR) for continuous data

*: Estimated effect size: OR for categorical variables (based on logistic regression model) and mean difference (based on linear regression model)

**: Details for all definitions are presented in the chapter appendix. ANS = autonomic nervous system

†: p< **0.**001, &: p<0.05

General manifestations: Patients in the EV-A71 group were significantly older than in the CV group (median 21 vs. 16 months, $p < 0.001$), but this may be influenced by the untyped EV that were probably EV-A71 during the peak of the 2011 season. The median illness day when patients were generally admitted to hospital was Day 3, but significantly earlier (Day 2) in the CV group compared to the EV-A71 group. Parents may have worried more about the younger children, especially during the second year of the outbreak when there was a high degree of public anxiety. In total 58% of the cases came from urban areas; those with EV-A71 infection were equally distributed between urban and rural areas however, while the proportion of the CV cases living in urban areas was higher, (103/167, 62%) compared to EV-A71 group (232/448, 52%) with OR of 1.50 (1.04, 2.16), p=0.028).

Almost all patients presented with fever, i.e. rectal temperature above 37.5^0C, but only 179/1203 (15%) had a rectal temperature of 40^0C or more, with equal frequency in the serotype comparison. However, presentation with skin lesions only, i.e with rash/vesicles but without mouth ulcers, was clearly associated with EV-A71 infection ($p< 0.001$). In contrast, presentation with mouth ulcers only without skin lesions was strongly associated with CV infection ($p< 0.001$) (Table 3.2). Other general symptoms, such as vomiting and diarrhea, occurred in only a small number of patients overall, with similar frequency in the EV-A71 and CV groups.

Central nervous system involvement: Myoclonic jerks were common, observed in 450/1203 (37%) of children overall, but were more frequent in the EV-A71 group (163/448, 37%) than the CV group (34/167, 21%) (OR= 2.17 (1.43, 3.35), $p < 0.001$). Drowsiness and focal neurological signs were quite uncommon at this time. Lumbar puncture was only performed in 79 cases in total, most of them were done during the first 9 months of the outbreak in 2011, and in 49/79 (62%) showed typical features of viral meningitis with mild to moderate CSF pleocytosis but normal protein and sugar levels.

Respiratory manifestations: Tachypnea for age was noted in 204/1195 (17%) cases during the first 24 hours, and was also significantly more common in the EV-A71 group (99/ 441, 22%) than in CV group (7/163, 4%) with OR of 6.45 (3.14, 15.59) $p<0.001$.

Irregular breathing was observed in 132/1190 (11%) cases in total, of which most were in the EV-A71 group, 83/441 (19%), compared to 3/163 (2%) cases in the CV group (OR=12.36 (4.54, 50.91), p<0,001). Severe respiratory pattern abnormalities such as labored breathing, stridor, or Cheyne-Stokes respirations were uncommon at this time, without a clear relationship to the serotype group.

Cardiovascular manifestations: Tachycardia, defined as a heart rate over 150 beats/min after adjustment for fever, was present in 709/1211 (59%) cases, in similar proportions of the EV-A71 and CV groups. However, high blood pressure (SBP over the Vietnamese MoH Stage 1 threshold) was noted in 192/1143 (17 %) of cases, with a strong relationship with EV-A71 infection, (103/314, 33%) compared to the CV group (18/94, 15%) with OR= 2.06 (1.19, 3.72), p=0.009.

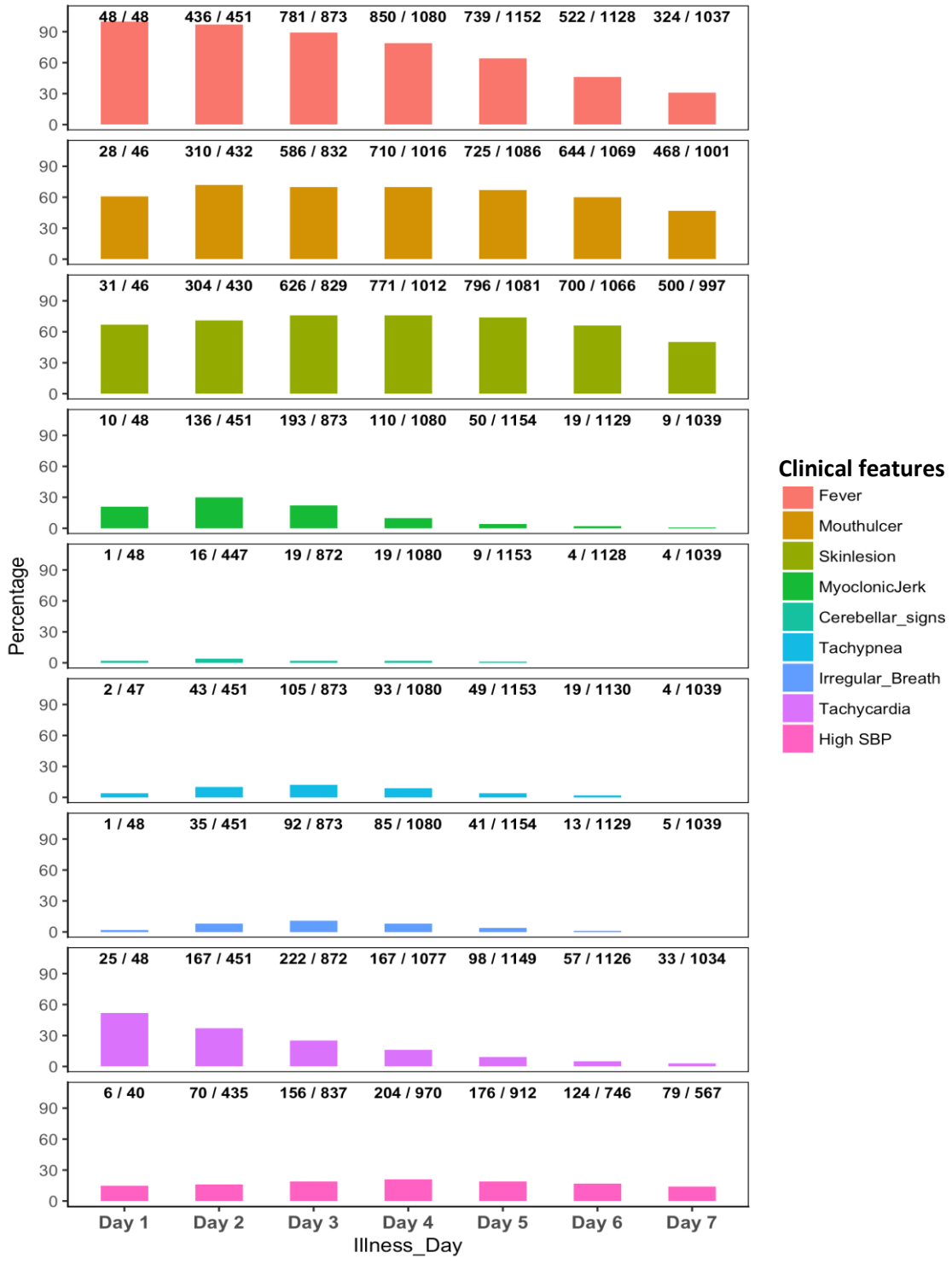
3.3.2 Evolution of clinical features over time, and development of complications

3.3.2.1 Progress in PICU – all cases

The patients were admitted to PICU on different days of illness, but in the majority, 832/1223 (67%), this was either Day 2 or Day 3. The progress of the various signs and symptoms after admission is shown in Figure 3.4 according to illness day. For most variables data were missing in less than 5% of cases over the time course. Blood pressure and respiratory distress were not recorded specifically in the hospital files in the late stages if they had been normal in the early stage and the patients were clinically stable. I reviewed these missing data and accepted that the data should be considered as normal.

Fever was noted in all cases seen on Day 1, and the proportion with fever gradually decreased over the first week. Skin lesions and mouth ulcer were also often noted on Day 1 and gradually disappeared in the following days. New skin or mouth lesions appeared after admission in only small numbers of patients, 48/1223 (4%) and 21/1223 (2%) of patients, respectively.

Figure 3.4: Progress of signs and symptoms over the first 7 days of illness in 1223 cases



* High SBP: SBP above Stage I of the Vietnamese MoH guidelines

Numerator: Numbers represent the number of patients with that feature that day /

Demoninator: the total number of patients with information for that day of illness

By contrast myoclonic jerks were only observed in 10/48 (20%) patients on Day 1, increasing to 30% on day 2, then decreasing over the followings days. Other neurological signs such as nystagmus or ataxia occurred rarely, but usually happened between Day 2 (16/447, 4 %) to Day 4 (19/1080, 2%).

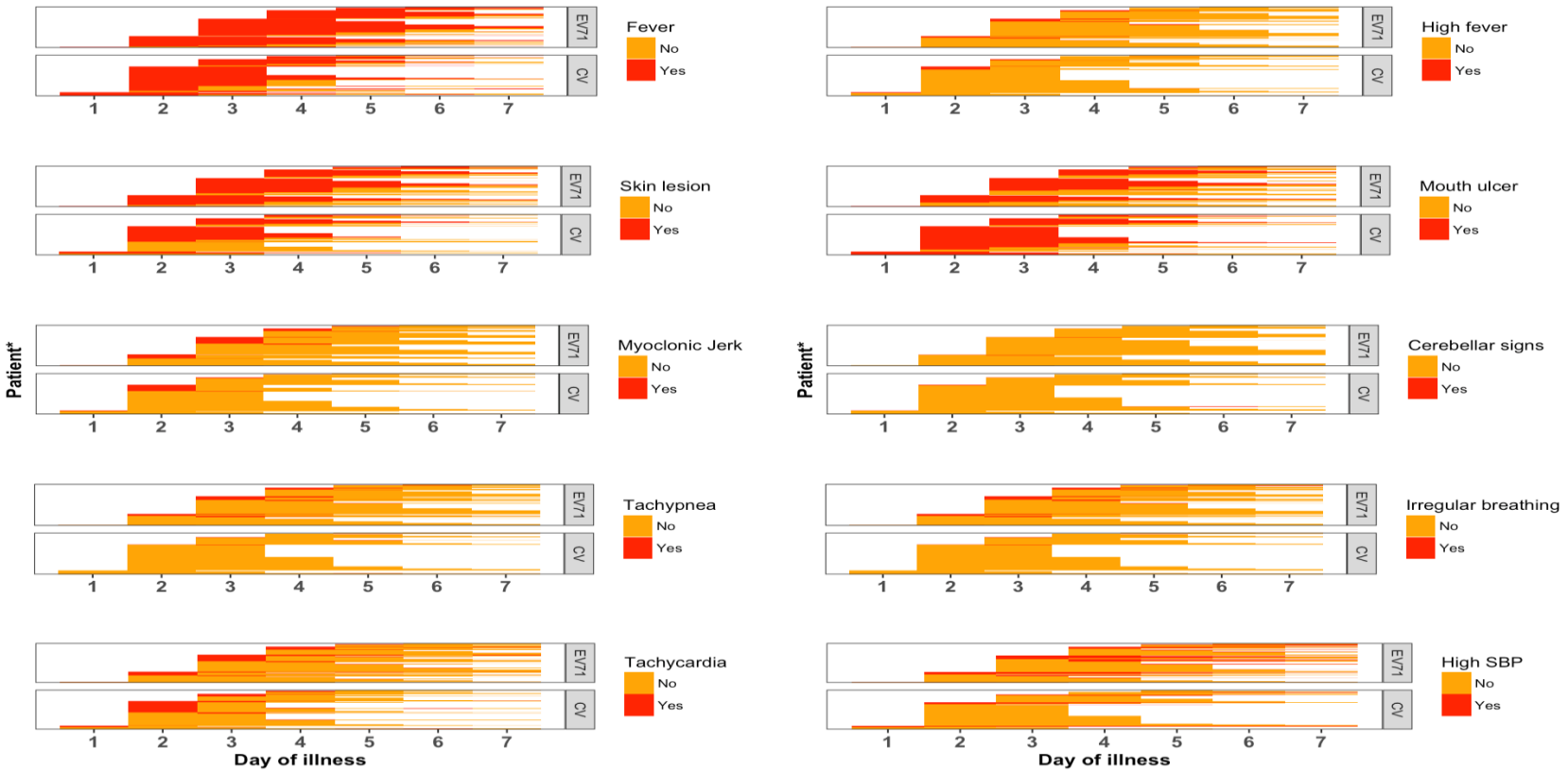
The earliest respiratory manifestation, i.e. tachypnea, occurred in a few cases on Day 1 (2/43, 2%) and Day 2 (43/ 451, 10%), but the greatest frequency was seen on Day 3 (105/873, 12%). Similarly, irregular breathing was highest on Day 3 (92/873, 11%) thereafter gradually reducing to Day 7 (5/1039, <1%). Severe respiratory distress, including laboured breathing, stridor, gasping respirations and apnea were rare, but occurred mostly on Day 3 (14/873, 2%). However, tachycardia occurred most commonly on Day 1 (25/48, 52%), then decreased progressively to Day 7 (33/1034, 3%). Meanwhile, High SBP increased from 6/40, 15% of cases on Day 1 to peak at 204/970, 21% of cases on Day 4, and was still present in 79/567 (14%) of cases on Day 7.

3.3.2.2 Clinical progress over time in the EV-A71 and CV groups

The progression of selected clinical features over time in the EV-A71 and CV groups is presented in Figure 3.5. The lasagna plot shows the presence or absence of each feature in individual patients (one line per patient) during the period of observation.

Visual inspection of these plots indicates that while fever was common in both groups during the early phase, it generally persisted for longer in the EV-A71 group. Skin lesions and mouth ulcers also persisted for longer in the EV-A71 group, although mouth ulcers were overall more common in the CV group during the first 3 days. Myoclonic jerks occurred more frequently and persisted for longer in the EV-A71 group. Similarly, tachypnea, irregular breathing, and High SBP all occurred more frequently and persisted for longer in the EV-A71 group than the CV group, but the two groups showed similar patterns for tachycardia.

Figure 3.5: Lasagne plots showing the progress of signs and symptoms over the first 7 days of illness in the EV-A71 and CV groups



*: Each patient is represented by a single line in the plot, with the presence or absence of each feature in that individual shown for each day under observation.

3.3.2.3 Overall outcomes and development of major complications (Table 3.3)

A total of 553/1223 (45%) of the children developed signs of brainstem encephalitis during their time in PICU, significantly more frequently in the EV-A71 group (198/448, 55%) than the CV group (42/167, 25%) with OR= 2.36 (1.60, 3.53), p<0.001. ANS dysregulation occurred in 211/1223 (17%) cases, and hypertension in 108/1223 (9%) cases, both strongly associated with EV-A71 infection (p< 0.001). Respiratory distress occurred in 182/1223 (15%) cases, and was also associated with EV-A71 infection. Clinical shock, pulmonary edema and cardiopulmonary failure occurred in 1 to 3% of the EV-A71 group, but did not occur at all in the CV group (Table 3.3). Focal neurological signs were rarely observed, recorded in only in 7/1223 (1%) cases across the groups. Neurological sequelae were noted at discharge in 9/1223 (1%) cases, but none of the children were followed up subsequently.

Overall, 10 children died during the study period, between May 2011 and January 2012. Details of the features noted within the first 24 hours on PICU in these cases, and a summary of complications that developed later, are presented in Table 3.4. The children ranged in age from 13 to 59 months, 50% were female, and disease progression was often rapid with four children dying within 24 hours of arrival. The remaining 6 children died 2-4 days later, in most cases with cardiopulmonary failure as the terminal event. EV-A71 was identified in 3 cases, in 2 of these cases with testing performed later on samples stored at the time of death. Five cases were positive with enterovirus species but did not have specific serotype identification performed, as this was not available at that time. Their samples were not also routinely stored for further serotyping later.

Table 3.3: Major complications and outcomes for 1223 PICU cases, presented according to viral serotype

	EV-A71 (N=448)	CV (N=167)	OR (95% CI)*	Other_EV (N=227)	NEG (N=381)
Major Complications					
Brainstem encephalitis	198 (55)	42 (25)	2.36 (1.60, 3.53)†	137 (60)	176 (46)
ANS dysregulation	110 (25)	12 (7)	4.20 (2.34, 8.25)†	36 (16)	53 (14)
Hypertension **	72 (16)	4 (2)	7.80 (3.17, 25.93)†	10 (4)	22 (6)
Clinical shock	6 (1)	0 (0)	#	3 (1)	3 (1)
Respiratory distress	108 (24)	3 (2)	17.36 (6.42, 71.27)†	38 (17)	33 (9)
Pulmonary edema	4 (1)	0 (0)	#	4 (2)	2 (1)
Cardiopulmonary failure	14 (3)	0 (0)	#	7 (3)	3 (1)
Overall Outcomes					
Death	3 (1)	0 (0)	#	5 (2)	2 (1)
Neurological sequelae	3 (1)	0 (0)	#	3 (1)	3 (1)
Hospitalization duration (days)	8.0 (7, 9)	7.0 (6, 9)	-0.43 (-0.95, 0.09)	8 (7, 9)	8.0 (7, 10)
Severe disease (Grade >=3)	162 (36)	7 (4)	0.08 (0.03, 0.16)†	68 (30)	70 (18)

Summary statistic is absolute count (%) for categorical variables and median (IQR) for continuous data

*: Odds ratio and p-values are based on logistic regression model; †: p< **0.**001

**: Systolic blood pressure above Stage 2 of the Vietnamese MoH guidelines.

#: cannot be compared because there were no events in one group

Table 3.4: Clinical features observed within the first 24 hours on PICU, and later progression, in the 10 children who died

No	Date	Sex	Age (mths)	PICU DOI	Signs presenting during first 24 hrs in PICU						Complications					Other notes	Enterovirus serotypes
					Location of lesion	T > 40°C	HR> 150 bpm	Tachy-pnea	Irregular breathing	Myoclonic Jerks	BE	HTN	Shock	Respiratory Distress	Pulmonary Edema		
1	May 11	F	37	Day 3	Skin & Mouth	(+)	(+)	(-)	(-)	(+)	(+)	(-)	(+)	(+)	(+)	Died at D5	EV-A71
2	Jun 11	F	19	Day 6	Skin	(-)	(+)	(+)	(+)	(-)	(+)	(+)	(-)	(+)	(-)	Died at D 10	Untyped EV
3	Jun 11	M	59	Day 3	Skin & Mouth	(-)	NA	(+)	(-)	(-)	(-)	(-)	(+)	(+)	(-)	Rapid progress < 24hrs	EV-A71
4	Jun 11	F	44	Day 2	Skin	(-)	(-)	(-)	(-)	(-)	(+)	(+)	(+)	(+)	(+)	Died at D4	Untyped EV
5	Jul 11	M	46	Day 2	Skin & Mouth	(+)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	Died at D5	NEG
6	Jul 11	M	13	Day 3	Skin	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(-)	(+)	(+)	Died at D6	Untyped EV
7	Jul 11	F	21	Day 4	Skin & Mouth	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(+)	Rapid progress < 24hrs	Untyped EV
8	Aug 11	M	21	Day 3	Skin	(+)	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(+)	Died at D5	Untyped EV
9	Oct 11	F	27	Day 4	Skin & Mouth	(+)	(+)	(+)	NA	(-)	(+)	(-)	(+)	(+)	(+)	Mottled skin (+), rapid progress < 24hrs	EV-A71
10	Jan 12	M	43	Day 3	Skin & Mouth	(-)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)	(+)	Rapid progress < 24hrs	NEG

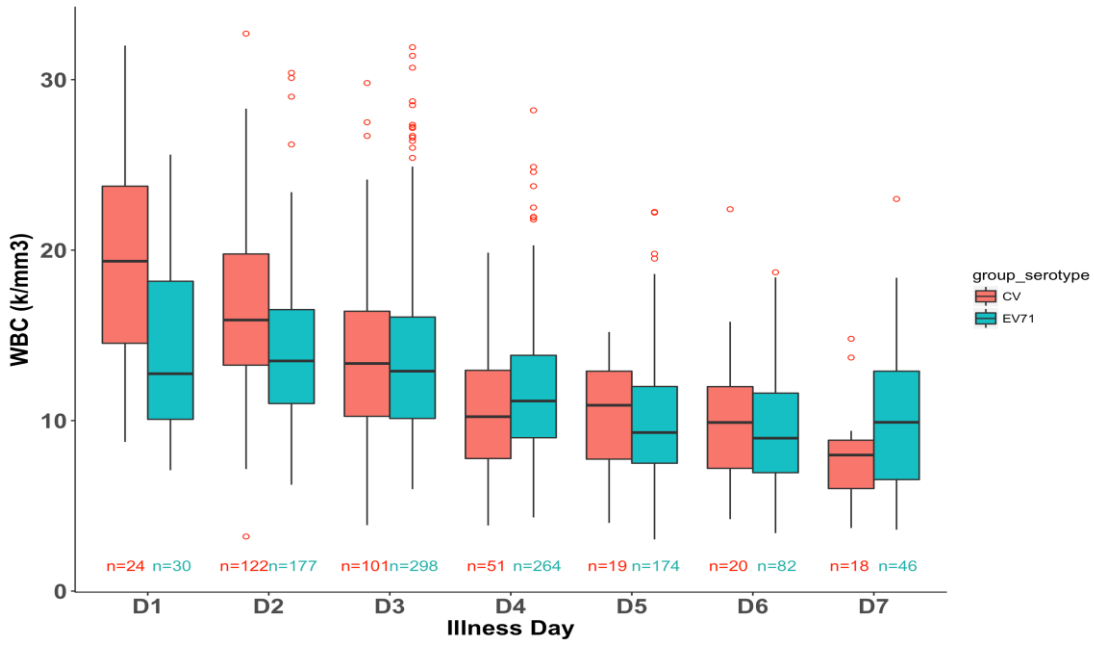
Abbreviation: DOI: Day of illness at admission, BE: Brainstem encephalitis, HR: heart rate, HTN: hypertension

3.3.3 Laboratory investigations in the 1223 cases

Results for standard laboratory investigations were incomplete for many patients. A full blood count was performed in most patients (1136/1223, 93%) during the first 24 hour in PICU, but other tests were performed according to clinical need rather than systematically.

The median (IQR) maximum white blood count (WBC) was 14 (11, 18) x10^9/L, and this was higher in the CV group compared to the EV-A71 group within the first 24 hours (Figure 3.7, Table 3.5). There was a progressive decline in values obtained on later days of illness, and since the CV infected patients were admitted a little earlier (median illness Day 2) than the EV-A71 infected patients (median illness Day 3), the interpretation of these data remain unclear. Otherwise, the proportion of thrombocytosis (platelet count > 400.000/mm3) was higher in EV-A71 group compared to the CV group [197/448 (44%) vs. 53/166 (32%), p=0.009], but the actual differences were small.

Figure 3.6: Boxplots showing white blood counts in the EV-A71 and CV groups, by illness day



n= number of results available for that serotype each day

Table 3.5: Laboratory investigations in the first 24 hours in PICU

Characteristics	n	EV-A71 (N=448)	n	CV (N=167)	Estimated effect (95% CI)*	n	Other EV (N=227)	n	NEG (N=381)
WBC ($x10^9$/L)	421	13.3 (10.6, 16.8)	151	16.1 (12.9, 19.5)	-2.6 (-3.5, -1.6) †	211	13.5 (11.2, 16.8)	353	12.4 (9.4, 16.0)
Pleocytosis (>16 $x10^9$/L)	421	122 (29)	151	76 (50)	0.40 (0.27, 0.59) †	211	63 (30)	211	87 (25)
Platelet ($x10^9$/L)	422	370 (310, 442)	151	334 (284, 396)	38 (17, 60)†	211	353 (296, 412)	353	329 (265, 401)
Hyperglycemia (>150 mg/dl)	256	5 (2)	109	2 (2)	1.07 (0.23, 7.52)	126	5 (4)	194	7 (4)
Serum Na (mmol/l)	126	134 (132, 136)	47	133 (130, 136)	1 (0, 2) &	113	136 (133, 138)	145	134 (132, 135)
Hyponatremia (<130 mEq/l)	126	10 (8)	47	9 (19)	0.36 (0.14, 0.98) &	113	8 (7)	145	12 (8)
CK-MB (UI/l)	33	32 (24, 42)	3	14 (14, 27)	19 (-13, 50)	19	35 (28, 60)	8	38 (28, 66)
Abnormal CKMB (>60 UI/l)	33	5 (15)	3	0 (0)	#	19	5 (26)	8	2 (25)
Troponin (pg/ml)	35	15.0 (7.5, 28.5)	3	2.0 (2.0, 6.0)	0.30 (-1.5, 2.1.)	22	0.0 (0.0, 12.0)	13	0.0 (0.0, 10.5)
Abnormal Troponin I (> 75 pg/ml)	35	2 (6)	3	0 (0)	#		0 (0)		1 (8)

Summary statistic is absolute count (%) for categorical variables and median (IQR) for continuous data

*:Estimated effect: OR for categorical variables (based on logistic regression model) and mean difference (based on linear regression model); †: p< **0.**001, &: p<0.05

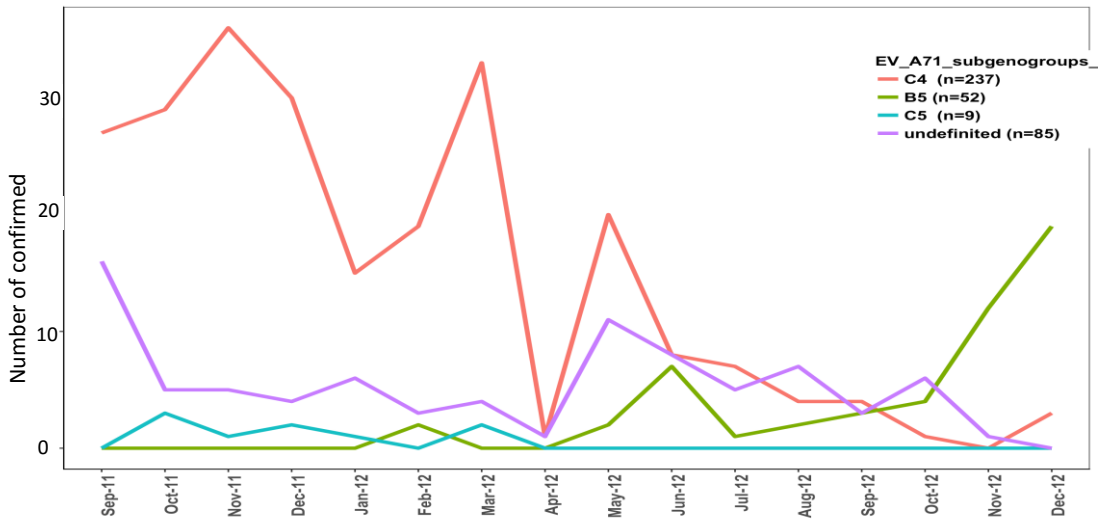
#: cannot be compared because there were no event in one group

Hyperglycemia (>150 mg%, random test)

3.3.4 Associations with EV-A71 subgenogroups

From the 448 EV-A71 positive patients, there were 383 samples available for further testing to identify the subgenogroups circulating from September 2011 to December 2012. However sequencing was unsuccessful in 85/383 (22%) of these cases, most likely because the viral load was low. Sequencing confirmed the presence of EV-A71 in the remaining samples. Genogroup C4 dominated overall, identified in 237/383 (62%) of the samples particularly during the latter part of 2011, gradually decreasing from April/May 2012 onwards (Figure 3.7). Meanwhile, genogroup B5 showed a clearly different pattern. It was found in 52/383 (14%) of cases, not at all in 2011, then with slowly increasing frequency during 2012 peaking in December of that year. Genogroup C5 was only identified in 9/383, (2%) of cases, circulating at low frequency in 2011 and disappearing from April 2012 onwards.

Figure 3.7: Distribution of EV-A71 subgenogroups from Sep 2011 to Dec 2012



Associations between EV-A71 genogroups and clinical features are presented in Table 3.6. Myoclonic jerks were noted within the first 24 hours significantly more often in the C4 genogroup (108/235, 46%) than the B5 genogroup (14/52, 27%), with an OR of 2.31 (1.21, 4.62), p=0.010. Other signs and symptoms were not notably different at this time, except that rare events like cerebellar signs and skin ANS features (such as mottling and profuse sweating) were only seen in patients infected with the C4 genogroup.

Table 3.6: Clinical features during the first 24 hours in PICU, and overall complications associated with subgenotypes of EV-A71 (n=383)

	C4 (N=237)	B5 (N=52)	OR (95% CI)*	C5 (N=9)	Undefined (N=85)
Selected signs and symptoms at admission					
Myoclonic jerks	99 (42)	13 (25)	2.18 (1.13, 4.45)&	4 (44)	31 (36)
Cerebellar signs	7 (3)	0 (0)	#	0 (0)	2 (2)
Tachypnea for age	56 (24)	11 (22)	1.12 (0.55, 2.42)	2 (22)	14 (16)
Irregular breathing	47 (20)	9 (18)	1.14 (0.54, 2.66)	3 (33)	10 (12)
Tachycardia (HR>150 bpm)	134 (57)	29 (56)	1.03 (0.56, 1.89)	6 (67)	53 (62)
High SBP (mm Hg)**	56 (25)	13 (28)	0.87 (0.44, 1.81)	4 (50)	11 (13)
Skin ANS features	4 (2)	0 (0)	#	0 (0)	1 (1)
Complications during the illness course					
Brainstem encephalitis	163 (69)	24 (46)	2.57 (1.40, 4.76) &	6 (67)	44 (52)
Paralysis	3 (1)	0 (0)	#	0 (0)	0 (0)
ANS dysregulation	67 (28)	9 (17)	1.88 (0.91, 4.32)	3 (33)	11 (13)
Hypertension***	49 (21)	9 (17)	1.25 ((0.59, 2.88)	2 (22)	4 (5)
Shock	4 (2)	0 (0)	#	0 (0)	0 (0)
Respiratory Distress	66 (28)	15 (29)	0.95 (0.50, 1.89)	3 (33)	8 (9)
Pulmonary Edema	2 (1)	0 (0)	#	0 (0)	0 (0)
Cardiopulmonary failure	11 (5)	0 (0)	#	1 (11)	0 (0)

Summary statistic is absolute count (%) for categorical variables.

*: Odds ratio and p-values are based on logistic regression model; &: p<0.05

#: cannot be compared because there was no events in one group

**: Systolic blood pressure above Stage 1 of the Vietnamese MoH guidelines. Missing blood pressure (5%), which could not be measured in some young children when distressed/crying.

***: Systolic blood pressure above Stage 2 of the Vietnamese MoH guidelines

Considering the whole time-course, brainstem encephalitis occurred significantly more often in the C4 genogroup (163/237, 69%) than in the B5 genogroup (24/52, 46%) with an OR of 2.57 (1.40, 4.76), p= 0.002, and major life-threatening complications including shock, pulmonary edema and cardiopulmonary failure were only seen in the C4 group. One child in the C4 genogroup died, but all other children in the groups selected for this analysis survived.

3.3.5 Risk factors for progression to severe disease

During the first 24 hours in PICU, most patients had signs and symptoms of moderate severity, but in some cases there was already evidence of disease progression, with severe manifestation such as respiratory distress or high blood pressure present at this time (Table 3.2). Among the 1272 cases potentially eligible for analysis, 68 (5%) progressed to one of the pre-defined severe outcomes within the first 24 hours on PICU, and were therefore excluded. Subsequently 58/1272 (5%) and 22/1272 (2%) children progressed to a severe outcome, on days 2 and 3 in PICU respectively, with one further case progressing on day 4. Most of these children eventually made a full recovery, apart from the 10 deaths previously described.

Excluding the cases that progressed quickly, there were 1204 cases that did not develop one of the severe outcomes during the first 24 hours, and these cases were selected for the multiple regression. Among them, 81/1204 (7%) cases did progress later, after more than 24 hours, while the remaining 1123 cases never progressed. From the original list, 9 clinical signs and symptoms or easily available laboratory parameters were selected as candidate predictors to assess in the multivariable analysis (Table 3.7). In the univariable regression, presence of skin lesions, tachypnea, and irregular breathing were strongly associated with disease progression and there was a borderline association for myoclonic jerks. Tachypnea for age and irregular breathing are closely related - I selected tachypnea for the multivariable regression as this is usually noted first, while irregular breathing is one factor that may prompt clinicians to consider ventilation (i.e. it is closer to one of the outcomes being assessed in the model).

In the multivariable regression, the findings with and without imputation for missing values were very similar (Table 3.7). Presence of skin lesions (OR=3.53 (1.61, 9.32), p<0.001) and tachypnea for age (OR=2.23 (1.26, 3.82), p=0.007) were the only two candidate predictors independently associated with disease progression in the main analysis. On repeating the analysis including EV-A71 status (Table 3.8), I confirmed the previous finding of a strong association between EV-A71 infection and presence of skin lesions.In this analysis, EV-A71 status was the strongest predictor for disease progression (OR=2.61 (1.33, 5.34), p=0.005), but skin lesions were no longer included in the final model.

Table 3. 7: Multiviariable logistic regression to identify risk factors for progression to severe outcome

Candidate predictors**	Severe outcome*				Logistic Regression								
	No (N=1123)		Yes (N=81)		Univariable			Multivariable			Multivariable (imputation)		
	n	Summary statistic	n	Summary statistic	OR	95% CI	Pvalue	OR	95% CI	Pvalue	OR	95% CI	Pvalue
Age (months)	1118	18 (12, 27)	81	17 (11, 25)	1.00	0.99, 1.02	0.581	1.01	0.99, 1.02	0.566	1.01	0.99, 1.02	0.412
Sex (F)	1123	427 (38)	81	35 (43)	0.81	0.51, 1.28	0.357	0.89	0.54, 1.51	0.672	0.86	0.54, 1.38	0.539
Illness day at PICU admission	1123	3 (2, 4)	81	3 (2, 3)	0.91	0.74, 1.11	0.354	0.92	0.72, 1.16	0.485	0.87	0.69, 1.08	0.203
Skin lesions	1071	798 (75)	74	69 (91)	3.37	1.64, 8.15	**<0.001**	3.53	1.61, 9.32	**<0.001**	3.37	1.62, 8.20	**<0.001**
High Fever (> 40^0C)	1108	157 (14)	75	16 (21)	1.64	0.89, 2.86	0.106	1.50	0.74, 2.86	0.250	1.56	0.84, 2.76	0.153
Tachycardia (HR > 150 bpm)	1114	642 (58)	79	52 (66)	1.42	00.88, 2.32	0.236	1.27	0.75, 2.21	0.380	1.49	0.91, 2.49	0.115
Myoclonic jerks	1105	385 (35)	79	36 (46)	1.56	0.98, 2.47	0.059	1.45	0.86, 2.41	0.159	1.49	0.93, 2.37	0.098
Tachypnea for age	1101	165 (15)	76	24 (32)	3.22	1.86, 5.39	**<0.001**	2.23	1.26, 3.82	**0.007**	2.23	1.31, 3.68	**0.004**
Pleocytosis (>16k/mm3)	1045	316 (30)	75	19 (25)	0.78	0.45, 1.32	0.363	0.86	0.47, 1.50	0.594	0.74	0.42, 1.26	0.269
Urban address	1123	642 (57)	81	52 (64)	1.34	0.85, 2.17	0.213						
Mouth ulcers	1090	788 (72)	74	52 (70)	0.91	0.55, 1.55	0.709						
Skin ANS features	1103	31 (3)	76	2 (3)	0.93	0.15, 3.17	0.926						
Irregular breathing	1097	96 (9)	76	20 (26)	3.72	2.10, 6.38	<0.001						
Cerebellar signs	1104	54 (5)	78	3 (4)	0.77	0.10, 2.06	0.652						
Thrombocytosis (>400k/mm3)	1046	320 (31)	75	26 (35)	1.20	0.73, 1.95	0.465						

*: Severe outcome was defined as death, shock requiring inotropes or fluid resuscitation, severe respiratory distress requiring ventilation, hypertension requiring milrinone, or high refractory fever requiring hemofiltration. Patients with any of these outcomes within 24 hours of PICU admission were excluded.

**: All predictors were assessed within the first 24 hours after PICU admissi

Table 3.8: Multiviariable logistic regression to identify risk factors for progression to severe outcome, including EV-A71 status

Candidate predictors	Severe outcome				Logistic Regression								
	No (N=936)		Yes (N=59)		Univariable			Multivariable			Multivariable (imputation)		
	n	Summary statistic	n	Summary statistic	OR	95% CI	Pvalue	OR	95% CI	Pvalue	OR	95% CI	Pvalue
Age (months)	932	18 (12, 27)	59	20 (11, 29)	1.01	(0.99, 1.03)	0.301	1.01	(0.99, 1.03)	0.489	1.01	(0.99, 1.03)	0.491
Sex (F)	936	584 (62)	59	33 (56)	0.77	(0.45, 1.31)	0.325	0.91	(0.50, 1.69)	0.756	0.81	(0.47, 1.41)	0.457
Illness day at PICU admission	936	3 (2, 4)	59	3 (2, 3)	0.97	(0.76, 1.22)	0.822	0.97	(0.72, 1.29)	0.858	0.9	(0.68, 1.17)	0.432
Skin lesions	895	645 (72)	55	48 (87)	2.66	(1.27, 6.51)	0.008	1.84	(0.77, 5.10)	0.178	1.86	(0.84, 4.74)	0.132
High fever (> 40^0C)	926	129 (14)	55	12 (22)	1.72	(0.85, 3.26)	0.126	1.43	(0.59, 3.09)	0.404	1.73	(0.83, 3.36)	0.136
Tachycardia (HR > 150 bpm)	930	530 (57)	57	39 (68)	1.64	(0.94, 2.97)	0.085	1.32	(0.70, 2.58)	0.393	1.62	(0.90, 3.01)	0.107
Myoclonic jerks	920	312 (34)	58	27 (47)	1.7	(0.99, 2.89)	0.054	1.52	(0.82, 2.78)	0.181	1.61	(0.92, 2.78)	0.093
Tachypnea for age	916	129 (14)	55	17 (31)	2.73	(1.46, 4.91)	0.002	1.93	(0.96, 3.71)	**0.066**	2.06	(1.09, 3.75)	**0.027**
Pleocytosis (>16k/mm3)	867	271 (31)	55	12 (22)	0.61	(0.31, 1.15)	0.129	0.71	(0.34, 1.39)	0.323	0.57	(0.28, 1.10)	0.094
EV-A71 positive	936	370 (40)	59	40 (68)	3.22	(1.86, 5.76)	<0.001	2.61	(1.33, 5.34)	**0.005**	2.56	(1.41, 4.80)	**0.002**
Urban address	936	534 (57)	59	38 (64)	0.97	(0.53, 1.84)	0.915						
Mouth ulcers	911	664 (73)	54	39 (72)	1.36	(0.80, 2.40)	0.264						
Skin ANS features	923	21 (2)	55	1 (2)	0.8	(0.04, 3.91)	0.819						
Irregular breathing	915	79 (9)	55	13 (24)	3.28	(1.63, 6.21)	0.001						
Cerebellar signs	911	30 (3)	57	1 (2)	0.52	(0.03, 2.52)	0.487						
Thrombocytosis (>400k/mm3)	868	266 (31)	55	20 (36)	1.29	(0.72, 2.26)	0.38						

Table 3.9: Multivariable logistic regression for severe outcome, focused on the groups with skin lesions only and mouth ulcers only

Candidate predictors	Severe outcome				Logistic Regression					
	No (N=1123)		Yes (N=81)		Univariable			Multivariable		
	n	Summary statistic	n	Summary statistic	OR	95% CI	Pvalue	OR	95% CI	Pvalue
Age (months)	1118	18 (12, 27)	81	17 (11, 25)	1.00	(0.99, 1.02)	0.58	1.01	(0.99, 1.02)	0.466
Sex (F)	1123	427 (38)	81	35 (43)	0.81	(0.51, 1.28)	0.357	0.90	(0.54, 1.51)	0.672
Illness day at PICU admission	1123	3 (2, 4)	81	3 (2, 3)	0.91	(0.74, 1.11)	0.354	0.93	(0.73, 1.16)	0.485
Skin lesions only	1071	241 (22)	74	22 (29)	1.44	(0.84, 2.37)	0.180	1.10	(0.61, 1.93)	0.735
Mouth ulcers only	1090	231 (21)	74	5 (7)	0.26	(0.09, 0.59)	**<0.001**	0.32	(0.11, 0.77)	**0.008**
High Fever (> 40^0C)	1108	157 (14)	75	16 (21)	1.64	(0.89, 2.86)	0.106	1.42	(0.70, 2.68)	0.322
Tachycardia (HR > 150 bpm)	1114	642 (58)	79	52 (66)	1.42	(00.88, 2.32)	0.236	1.27	(0.74, 2.20)	0.386
Myoclonic jerks	1105	385 (35)	79	36 (46)	1.56	(0.98, 2.47)	0.059	1.37	(0.82, 2.29)	0.226
Tachypnea for age	1101	165 (15)	76	24 (32)	2.62	(1.55, 4.32)	**<0.001**	2.26	(1.28, 3.88)	**0.006**
Pleocytosis (>16k/mm3)	1045	316 (30)	75	19 (25)	0.78	(0.45, 1.32)	0.363	0.87	(0.48, 1.52)	0.644
Urban address	1123	642 (57)	81	52 (64)	1.34	(0.85, 2.17)	0.213			
Skin ANS features	1103	31 (3)	76	2 (3)	0.93	(0.15, 3.17)	0.926			
Irregular breathing	1097	96 (9)	76	20 (26)	3.72	(2.10, 6.38)	<0.001			
Cerebellar signs	1104	54 (5)	78	3 (4)	0.77	(0.10, 2.06)	0.652			
Thrombocytosis (>400k/mm3)	1046	320 (31)	75	26 (35)	1.20	(0.73, 1.95)	0.465			

Tachypnea for age persisted in the model with imputation for missing values, with OR of 2.06 (1.09, 3.75), p=0.027, but with borderline significance in the standard model (OR=1.93 (0.96, 3.71), p=0.066).

In the light of this finding and given the previous analysis showing different relationships for skin lesions and mouth ulcers alone with enterovirus serotype, I also ran a post-hoc analysis focused on patients with either of these features separately. The presence of mouth ulcers without skin lesions was protective with OR=0.32 (0.11, 0.77), p=0.008 (Table 3.9), but skin lesions alone did not come out in the multivariable analysis.

3.4 Discussion

In this chapter I presented a detailed description of the clinical features of 1272 HFMD cases that were managed on our PICU over a 2 year period during the recent major outbreak in Ho Chi Minh City. I assessed over 98% of all the children admitted with a clinical diagnosis of HFMD in the acute phase (<7 days of illness). A small number of children initially admitted for observation were not included in the review as the PICU clinician's final assessment was of Grade 1 or 2a disease and they were soon discharged back to the pediatric wards. Although this was a retrospective file review, the data were quite comprehensive, generally with less than 5% missing information for all the variables assessed. In part this reflects the strict adherence to MoH guidelines for observation and management of HFMD cases that were drawn up during the early phase of the outbreak; with this good recordkeeping I feel that the data are representative of the progression of HFMD clinical features despite the inevitable limitations of a retrospective study.

Young children under 3 years of age were affected primarily, with similar age ranges found among the patients with mild disease (HTD population) and those with more severe symptoms who were admitted to PICU. Both EV-A71 and CV-A16 were reported as circulating in southern Vietnam between 2005 and 2011 [89, 201], potentially providing a degree of immunity in the community, especially among the older age

groups [200], while younger children remained susceptible to infection with emerging serotypes [210]. Serotype identification was not done in the early stage of the outbreak in 2011 as the necessary serotype specific PCR was not available at that time and specimens were not routinely stored, but given the temporal evolution of the EV-A71 related cases after September 2011 when the serotype specific PCR was introduced, it seems very likely that many of the cases during the summer months of 2011 were also EV-A71 related. The subsequent transition from EV-A71 to CV circulation during 2012 may explain the changing ratio of severe HFMD cases compared to overall HFMD cases in HTD that we found between 2011 and 2012 (Figure 3.3) [211].

Typical neurological signs and symptoms previously observed in severe HFMD cases indicate involvement of the brainstem [82, 83]. In this study, the analysis of longitudinal data allows the progression of moderate and severe manifestation to be examined more clearly. Fever is commonly the first symptom, with skin lesions and mouth ulcer often occurring concurrently with fever in the early phase, sometimes persisting up to the 7th day of illness. Neurological signs, including myoclonic jerks and cerebellar signs were rare on the first day but did occur on the second and third days, a pattern consistent with theoretical ideas on enterovirus pathogenesis [64]. Subsequently other manifestations of CNS involvement, including tachypnea and irregular breathing or respiratory distress were mainly seen from day 3 of illness and lasted for around 3 days. Similarly cardiovascular abnormalities, other than tachycardia which was often present from day 1, commonly became apparent between days 3-5 of disease. Many other factors may contribute to tachycardia, including fever (although this was adjusted for), and distress due to mouth ulcers/skin lesions, inability to swallow etc. during the early phase. Development of hypertension from day 3 onwards may indicate viral spread to involve the medulla oblongata [134], but may also implicate an aggressive systemic inflammation response [64]. Brainstem involvement can result in an imbalance in the sympathetic/parasympathetic control mechanisms, potentially leading to a sympathetic storm, especially in association with EV-A71 [64].

EV-A71 has been considered as the primary cause of severe HFMD manifestations in the Asian epidemics over the last 10-20 years, with 10-30% of hospitalized cases

reported to progress to CNS complications, including meningitis, polio-like paralysis, and brainstem encephalitis [68]. In line with other reports [104, 207, 212], in this study I found the range of severe signs and symptoms to be more common in those with confirmed EV-A71 infection, compared to among the children infected with a variety of CV serotypes; myoclonic jerks, respiratory abnormalities, hypertension and other ANS signs were all more common and persisted for longer in the EV-A71 group. However, although most of the severe complications occurred in patients infected with EV-A71, problems also occurred in some children infected with CV, albeit with a lower frequency. Brainstem encephalitis (25%), hypertension (8%) and severe respiratory distress (2%) were all documented in the CV group, but none of these cases progressed to develop pulmonary edema or cardiopulmonary failure.

The variety of clinical manifestation of HFMD observed in different studies likely depends on the neurotropic potential of the enterovirus serotype [213, 214], as well as on the host immune response [215, 216]. Within the EV-A71 serotype, there is also evidence to suggest that neurovirulence varies according to the specific genogroup involved [64, 217]. Genogroups C1 and C4 were reported to be circulating in Vietnam in 2005, later shifting to C5 in the latter half of 2005 and beyond [83, 89]. Detailed analysis of the circulating EV-A71 genogroups from the patients described here has shown that C4 dominated in 2011, with evidence of a shift to B5 in 2012. Associations between disease severity and EV-A71 genogroups have been difficult to clarify because of limited capacity to perform genogroup identification in many countries at risk of HFMD outbreaks, and also differences in study designs between countries or over time, thus limiting comparability of data. However group C2 in the outbreak in Western Australia [218], and B4 in the outbreak in Sarawak, Malaysia [192], were linked to severe neurological manifestations of HFMD. Although data collection in this study was retrospective, the data quality was generally good and outcome definitions were applied systematically to the whole dataset; comparative analysis showed that C4 was significantly more likely to cause brainstem encephalitis than B5, and that other severe complications also occurred either exclusively in the C4 group or more frequently in this group than the B5 group, indicating that genogroup C4 viruses are particularly neurotropic.

For the risk factor analysis I first carefully defined the severe outcomes of interest, and also made sure that any children in whom these events occurred within the first 24 hours in PICU were excluded (68 children), so that the analysis focused on identifying factors that predicted events occurring later in the disease evolution, rather than associations that were temporally related but not actually predictive. For the candidate predictors I was also careful to exclude variables that were too closely related to the outcomes selected. A total of 81 children experienced one or more of the severe outcomes at least 24 hours after PICU admission, thus allowing inclusion of 8-9 variables in the regression model. I selected candidate predictors occurring within the first 24 hours, either that have been previously published as showing an association with severe disease [112, 194, 202-209], or that were based on our clinical experience at HTD. However, although duration of fever for more than 3 days has been found to be associated with severe disease before, since I wished to look at factors present at admission and most cases were admitted on day 2 or 3 and progressed (if at all) within the next 48-72 hours this could not be included.

A number of the candidate predictors that have previously been shown to be associated with severe disease, e.g. age, sex, high fever, myoclonic jerks, tachycardia, were not significant in the univariable analysis and did not come through in the multivariable analysis after controlling for other factors. Presence of skin lesions and tachypnea for age were the only two candidate predictors that were independently associated with subsequent deterioration, and both showed strong relationships with the outcome. Further analysis showed that the presence of skin lesions was related to EV-A71 status; this was not included in the original multivariable model as I was interested in readily available signs and symptoms or lab tests, and serotype specific PCR is unlikely to be available in most countries where major epidemics of severe HFMD occur. However, given the strong relationship with skin lesions this may be taken as a proxy for EV-A71 infection in this analysis. Presence of skin lesions is quite a broad category however, and it may be that in other epidemics when different serotypes prevail that also cause skin lesions but may be less neurotropic, that the relationship would not be maintained.

On the other hand, presence of mouth ulcers alone, without skin lesions, was related

to CV infection, and in general the severe outcomes that I was interested in predicting did not occur in the CV group. In a meta-analysis of studies mostly conducted in mainland China [112], patients who presented with oral involvement were not at higher risk of severe disease, although in an evaluation of patients presenting with skin lesions with or without oral involvement in a case-control study in Singapore [205], oral lesions were associated with severe disease. In a post-hoc analysis I repeated the multivariable regression with presence of skin lesions and mouth lesions alone as candidate predictors, and found that presence of mouth ulcers without skin lesions had a strong protective effect, probably as a surrogate for CV infection. This may be of more use practically in outbreak situations, when deciding how to triage large numbers of children with potentially severe disease.

Tachypnea and irregular breathing were both associated with severe outcome in the univariable regression, consistent with brainstem involvement. For the multivariable regression model I selected tachypnea for age rather than irregular breathing for the reasons mentioned earlier, and this was independently associated with disease progression in the main analysis. After inclusion of EV-A71 status, the association was borderline, confirming a strong interaction between EV-A71 and respiratory complications.

The main limitation of this study was the fact that data collection was retrospective rather than prospective. However the practicality of collecting data prospectively during a major outbreak on this number of cases would be very daunting and would probably require a separate team of research staff so as not to compromise patient care. By making use of the MoH recommendations for monitoring and management of HFMD cases I was able to describe each patient's condition within the first 24 hours, but for the risk factor analysis I could only include outcome events occurring after the first 24 hours. Had it been possible to collect data prospectively it would have been theoretically feasible to use a shorter time-window, such as 12 hours, and so more of the events within the first 24 hours could have been included, potentially reducing bias and allowing additional candidate parameters to be assessed in the model.

Secondly by focusing the study on patients admitted to PICU this selected for children who already had some degree of compromise. However, the threshold for PICU

admission during this outbreak was relatively low due to heightened public anxiety, and I defined the outcomes for the risk factor analysis carefully, only including endpoints that were objective and likely to be accepted by all ICU clinicians as truly severe, rather than including more subjective features that could be open to interpretation. In consequence, the results from my analysis looking at longitudinal relationships between pre-defined factors and outcomes at least 24 hours later, differed from previously published studies, which did not clearly distinguish the timeline. Most other studies described associations with severe disease, often using less strict definitions for the endpoints. Of course in an outbreak situation the ability to triage before PICU admission would be a major advantage. But to look at risk prediction from an earlier stage in the illness course, assessments would have been needed on the whole suspected HFMD population in HTD, in order to identify factors that predicted the need for transfer to ICU. This is not practical for a detailed prospective study, but might be feasible if an electronic surveillance system could be set up, whereby a limited number of important features were recorded each day in the hospital patient database as part of routine clinical practice, ideally with the same system in operation at all the major hospitals across the region.

3.5 Conclusions

In summary, I have presented the clinical features of 1272 HFMD cases admitted to PICU during the 2011-2012 outbreak, in whom an enterovirus was identified using molecular methods in around 70% of cases, EV-A71 in 35%, various CVs in 13%, and echovirus/other serotypes in 18% of the cases. I described the characteristics on admission and the evolution of signs and symptoms over the first 7 days, comparing the findings between the EV-A71 and the CV groups. Neurological signs and symptoms occurred with greater frequency and persisted for longer in the EV-A71 group. Ten children died overall and severe complications occurred frequently, specifically signs consistent with brainstem encephalitis were found in 42% of children, commonly between days 3-5 of illness.

Although all severe manifestations were significantly associated with EV-A71 infection, they also occurred in patients infected with other enterovirus serotypes, including CVs

and echovirus. Brainstem encephalitis was more likely to occur in patients infected with the EV-A71 C4 subgenogroup than other genogroups. During an outbreak changes in the pattern of dominant viruses that are in circulation may result in emergence of more or less severe phenotypes over time.

In the risk factor analysis presence of skin lesions and tachypnea for age within 24 hours of PICU admission were the only two candidate predictors that were independently associated with subsequent deterioration to one of the pre-defined severe outcomes. Both these features showed an interaction with EV-A71 infection. Presentation with mouth ulcers alone, without skin lesions, was protective against the severe outcomes in the risk factor analysis, and was linked to CV infection. Patient who present with tachypnea or skin lesions should be closed observed to allow early recognition of progression to severe disease while patients with mouth ulcers only are unlikely to develop complications.

The burden of emerging enterovirus infections affecting young children in the Asia-Pacific region is considerable, with ongoing endemic transmission occurring in many countries, superimposed with major outbreaks every few years. The findings described here will be important in helping clinicians to assess and triage children with suspected HFMD, allowing appropriate follow-up and treatment to prevent severe complications of this common disease.

Appendix 1: Checklist for doctor and nurses to follow and manage suspected HFMD cases (Vietnamese MoH). Note that the frequency of observations depends on severity grade

Date /time **Signs/Symptoms**					
History					
Vomiting					
Myoclonic jerks (times)					
Irrititability / Sleepiness					
Limb tremor / ataxia					
Examination					
Rash / Mouth ulcer					
Heart rate (bmp)					
Respiratory rate (time/minute)					
BP (mmHg) – SBP/ DBP					
Temp (^{O}C)					
Myoclonic Jerk (times)					
Neurological focus signs (describe):					
Limb tremor / ataxia					
Local sweating (where?)					
Convulsion (**L**: Local, **G:** general					
Mottled skin (Local)					
Cold skin / CRT > 2 s					
Consciousness (**1**-Alert, **2**-Dowsiness, **3**-Coma)					
GCS					
Respiratory rales (crackle, bronchi, wheeze)					
WBC (x10^{9}/L)					
Glycemia (mg/dl)					
PCR (+: indication, EV, EV-A71, negative)					
Situation & Clinical stage					
Shock					
Respiratory distress					
Coma					
Pulmonary edema					
Clinical grade (currently)					
Follow up and management					
Oxygen/ intubation					
Dobutamine(µg/kg/m)					
……..(µg /kg/m)					
Milrinone (µg /kg/m)					
Gamma-globulin (dose 1 or 2)					
Sedation: Phenobarbital (mg)					
Mannitol (volume-rate)					
Antibiotic:					
Arterial catheter					
CVP (cmH20)					
Follow up duration (hour - minutes)					
Doctor /Nurse's Signature					

Appendix 2: Definitions for clinical signs and symptoms, complications and disease severity and outcomes

Variables	Type	Value	Definition
Demographic			
Age	Cat	Months	
Urban/Rural	Binary		Urban: district in city or town in provinces
Kindergarten	Binary	Yes/no	Studying in kindergarten
Members in family infected with HFMD	Binary	Yes/no	Any other member of family who was diagnosed with HFMD
Illness day	Cat	Days	The illness day of disease when admission
Underlying disease	Binary	Yes/no	Chronic disease
Previous HFMD infection	Binary	Yes/no	Have been suffered from HFMD before current infection
Signs and syndromes			
Fever	Binary	Yes/no	$T^0 > 37.5$,
High Fever	Binary	Yes/no	$T^0 > 40$.
Vesicle rash	Binary	Yes/no	Blister lesion examined by doctor
Mouth ulcer	Binary	Yes/no	Eruption lesion in the mouth examined by doctor
Cardiovascular system involvement			
Tachycardia	Binary	Yes/no	Any of heart rate > 150 bmp (after justify 10bmp per 1^oC above 37^oC
High Systolic Blood Pressure	Binary	Yes/no	Any of SBP is above MoH criteria (see in table 1.4, chapter 1)
Arrhythmia	Binary	Yes/no	Any event that recorded by doctors or presented in ECG recording
Mottled skin	Binary	Yes/no	Abnormal skin that covered with whitish spots and darkish color areas that recorded by doctor
Profuse Sweating	Binary	Yes/no	Local or general sweating that recorded by doctors of nurses in hospital files
Skin ANS features	*Binary*	Yes/no	Clinical HFMD and cold sweating and/or mottled skin
Respiratory distress			

Tachypnea	Binary	Yes/no	Respiratory Rate: >6≤12 mth: ≥ 50; >12≤72 mth: ≥ 40; >72 mth≤12 yrs: ≥ 30; >12 yrs: ≥ 25 that assessed by clinical doctor
Irregular breathing	Binary	Yes/no	Irregular pattern that recorded by doctors
Stridor	Binary	Yes/no	Abnormal inspiration sound that recorded by doctors.
Resp. retraction	Binary	Yes/no	Labor breathing
Wheezing	Binary	Yes/no	
Gasp breathing	Binary	Yes/no	
Cheyne-Stoke	Binary	Yes/no	Irregular breathing with short apnea period (<15s)
Apnea breathing	Binary	Yes/no	Apnea lasting over 15 seconds or gasp breathing recorded by doctor
CNS involvement			
Drowsiness	Binary	Yes/no	GCS <15 or stupor or legarthy manifestation that recorded by doctors
Irritability	Binary	Yes/no	Abnormal behavior that recorded by doctor or nurses
Myoclonic jerks	Binary	Yes/no	A jerk recorded by doctor
Cerebellar signs	*Binary*	Yes/no	Presented any of the following signs: limb tremor, nystasmus, ataxia
Limb weakness	Binary	Yes/no	Lack of strength of limbs recorded by doctor
Cranial nerve paralysis	Binary	Yes/no	Any cranial nerves paralysis recorded by doctor
Laboratory			
Pleocytosis	Binary	Yes/No	WBC > 16 $x10^9/L$
Thrombocytosis	Binary	Yes/No	Platelet > 400 $x10^9/L$
Hyperglycemia	Binary	Yes/No	Any blood sugar > 150 mg/dl
Hyponatremia	Binary	Yes/No	Serum Na< 130 mmol/dl
Complications			
Brainstem encephalitis	*Binary*	Yes/no	Clinical HFMD and present of myoclonus and/or ataxia and/or nystagmus and/or oculomotor palsies, and/or bulbar palsy and/or brainstem abnormality in MRI and/or abnormal respiratory pattern (irregular breathing, labor breathing and severe respiratory breathing)
Aseptic Meningitis	*Binary*	Yes/no	Clinical HFMD and cells/CSF >5 and bacterial culture (–ve)

Acute flaccid paralysis	*Binary*	Yes/no	Flaccid muscle weakness and lack of reflexes
Hypertension			SBP above Stage 2 of the Vietnamese MoH guidelines
ANS dysregulation	*Binary*	Yes/No	At least 2 of the following features: heart rate of 150-170 beats/min, systolic blood pressure variability with absolute values higher than threshold in MoH guideline, profuse sweating, mottled skin, respiratory abnormalities, and hyperglycemia.
Myocarditis	*Binary*	Yes/no	Cardiac injury and clinical cardiac failure and inotrops using (dobutamin or dopamin)
Shock	*Binary*	Yes/no	SBP < 70+ 2n and requirement of fluid resuscitation or vasopressor medicines
Pulmonary edema	*Binary*	Yes/no	Respiratory distress and tachycardia and tachypnea and/or rales and pink frothy secretion and bilateral pulmonary infiltrates without cardiomegaly in chest radiograph
Respiratory distress	*Binary*	Yes/no	Tachypnea for age and/or irregular breathing and/or stridor and/or apnea and/or gasp
Cardiopulmonary failure	*Binary*	Yes/no	Tachycardia (adjusted by fever) >150 bpm and respiratory distress and pulmonary edema and shock and/or pulmonary congestion on chest radiography and reduced cardiac contractility on echocardiography
Management			
IVIG	Binary	Yes/No	Patient received any dose of IVIG
MgSO4	Binary	Yes/No	Patient were commenced MgSO4 for hypertension
Nircardipine	Binary	Yes/No	Patient were commenced Nircardipine for hypertension treatment
Oxygenation	Binary	Yes/No	Patient received oxygen because of respiratory distress
Ventilation	Binary	Yes/No	Both non-invasive and invasive ventilation
Inotropes	Binary	Yes/No	Patient were commenced dobutamine or dopamine
Fluid resuscitation	Binary	Yes/No	Patient were commenced a bolus of fluid challenge or fluid resuscitation because of shock
Hemofiltration	Binary	Yes/No	Patient were performed the CVVH
Outcome			

Death	Binary	Yes/No	Both death or discharge because of worst condition
Neurological sequelae	Binary	Yes/No	Any neurological sequelae still exist at discharge
Duration of Hospitalization	Cat	Days	Time from HTD admission to discharge
Severe grade	Binary	Yes/No	Clinical HFMD and grade ≥2b at discharge

Appendix 3: Description of pre-defined candidate predictor present during the first 24 hours in PICU and definitions for severe outcome

Prognosis factors			
Factors	Type	Value	Definition
Age	Cat	Months	
Sex	Binary	M/F	
Weight	Cat	Kg	
Urban/Rural	Binary	Yes/no	See above
Illness day	Cat	Days	See above
High fever	Binary	Yes/no	See above
Skin lesion	Binary	Yes/no	See above
Mouth ulcer	Binary	Yes/no	See above
Tachycardia	Binary	Yes/no	See above
Skin ANS features	*Binary*	Yes/No	See above
Tachypnea	Binary	Yes/no	See above
Irregular breathing	Binary	Yes/no	See above
Myoclonic jerks	Binary	Yes/no	See above
Cerebellar signs	*Binary*	Yes/no	See above
Pleocytosis	Binary	Yes/No	See above
Thrombocytosis	Binary	Yes/No	See above
Hyperglycemia		Yes/No	See above
Severe outcome	Binary	Yes/No	Death or presenting severe condition that defined as either a) hemodynamic unstable that required fluid resuscitation or inotropes or b) severe respiratory distress that required ventilation support, or c) severe high blood pressure that required milrinone, or d) high refractory fever requiring hemofiltration,

Chapter 4

THE MAGNESIUM SULFATE TRIAL

4.1 Background

Neurological manifestations of enterovirus infections include a range of problems such as aseptic meningitis and acute flaccid paralysis, but the issue of particular concern in severe HFMD is brainstem encephalitis. ANS dysregulation may occur, potentially with rapid progression to cardiopulmonary failure [64]. Although the mechanisms underlying the ANS dysregulation have not been clearly defined there is evidence that inflammation occurs in the medulla oblongata and cervical spinal cord, causing increased sympathetic activity and resulting in severe systemic and pulmonary hypertension, and eventually pulmonary oedema [64]. The clinical features indicating ANS dysregulation include high persistent fever, profuse sweating, mottled skin, tachycardia, tachypnoea, hypertension and hyperglycaemia [83]. One potential mechanism could be related to high catecholamine levels [64, 111]. Alternatively, in a number of animal studies of neurogenic hypertension, associations have been demonstrated between inflammatory cytokine levels and enhanced vasomotor and cardiac sympathetic drive [120].

Management of ANS dysregulation presents particular challenges. In one report from Taiwan, use of the phosphodiesterase-3 inhibitor, milrinone, was said to control hypertension and support myocardial function in a group of 24 children with severe HFMD compared to historical controls [117]. In addition, milrinone has also been shown to decrease mortality in HFMD patients with pulmonary edema in a small open-label randomized clinical trial in Viet Nam [168]. This has now become the recommended therapy for severe HFMD with ANS dysregulation in Vietnam, with MoH Guidelines setting down indications for when milrinone should be commenced in suspected HFMD cases. Currently the MoH Guidelines define the intervention level for use of milrinone at a systolic pressure exceeding the 99th centile for age plus 5 mm Hg, which approximates to the internationally accepted definition of Stage 2 hypertension

in children (Appendices, page 198) [169]. However, clinical failures still occur despite high dose IV milrinone, and a number of children go on to require haemofiltration and ventilatory support, the next steps recommended in the MoH guidelines [170]. Secondly, there is very little clinical or safety data available with respect to milrinone use in children, apart from a few small studies following cardiac surgery. Adverse effects reported in adults include ectopic activity and potentially life-threatening ventricular arrhythmias [219]. Reports in children suggest that milrinone use is an independent risk factor for clinically significant tachyarrhythmias after congenital heart surgery, and may be associated with development of acute renal failure [220].

In 2011, during the first year of the major HFMD epidemic in Vietnam there were a significant number of milrinone treatment failures. As described in the introduction, MgSO4 is a drug in regular use on our PICU for children with ANS dysregulation in association with neonatal tetanus. Our experience of using this drug for neonatal tetanus has been very positive, so therefore I considered the possibility of using it as second line therapy for the severe HFMD cases. Formal safety data relating to use of $MgSO_4$ in paediatrics are limited, but from its use in children with severe asthma, and in a small number of neonates with uncontrolled pulmonary hypertension, adverse effects appear to be infrequent [176, 221, 222]. From the December of 2011 to the December of 2012, I used MgSO4 for a total of 18 severe HFMD patients that failed on milrinone, and only 2 of these cases required ventilation (2/18, 11%), in both cases because of respiratory distress, and there were no other serious adverse events (SAEs). By comparison, during 2011 before $MgSO_4$ was being used, of the 46 cases treated with milrinone alone, 13 required ventilation (13/46, 28%).

In summary, although HFMD had become one of the major contributors to childhood morbidity and mortality in Vietnam, in 2011 management strategies relied on guidelines that were based on expert opinion and only two small clinical studies [83, 117, 150, 168]. The efficacy of milrinone, an expensive drug with a significant toxicity profile, for management of ANS dysregulation was unclear, and $MgSO_4$, an alternative therapeutic agent that is cheap, safe and easily available appeared to be effective as second line therapy in severe cases. I hypothesized that intervention with MgSO4

early, when ANS dysregulation first becomes apparent, might control cardiovascular instability more effectively and prevent progression to severe disease. Therefore I developed a protocol for a randomized controlled intervention trial comparing MgSO4 with placebo in children with HFMD, autonomic instability and systemic hypertension. In collaboration with Professor Wills I was able to obtain a grant to support this trial from the Thrasher Research Fund, and also to obtain the necessary ethical permissions from the Vietnamese MoH and other regulatory bodies. Recruitment commenced in June 2014. However over the next two years the number of HFMD cases admitted to HTD declined dramatically and by the end of 2016 the trial had to be stopped on the grounds of futility.

In this chapter I will present information on the study design, execution, and findings from the trial. Additional information, including all definitions, standard operating procedures (SOPs) etc., is provided in Appendices. The trial was registered with ClinicalTrials.gov (NCT01940250, Registered 22 August 2013), and the full protocol has been published [223].

4.2 Materials and methods

4.2.1 Study design

This was a randomized, double-blind, placebo-controlled trial of IV $MgSO_4$ versus placebo in Vietnamese children with HFMD and signs of ANS dysregulation with systemic hypertension. Two study sites in Ho Chi Minh City were planned: the PICU at HTD as a single site initially; and the High Dependency Unit (HDU) on the Infectious Diseases Ward at CH1 to join later once all study procedures were running smoothly.

The study protocol and its associated documents were reviewed and approved by the ethical committees of HTD and CH1, the Oxford University Tropical Research Ethics Committee, and the Ethical Committee of the Vietnamese MoH.

All patients aged 6 months to 15 years with a clinical diagnosis of HFMD requiring PICU/HDU admission at these sites were eligible for enrolment into the study if they developed systemic hypertension and at least one other clinical sign of ANS dysregulation. Potential participants must also fulfil the indication of invasive BP

monitoring on clinical grounds. Trained study physicians assessed potential patients for the following inclusion and exclusion criteria, which are defined in more detail on page 202 of the Appendices.

4.2.2 Inclusion and exclusion criteria

The following criteria were applied: a) the SBP, measured via an indwelling catheter, exceeds the internationally recognized definition for Stage 1 hypertension in children (Appendices, page 198) [224]; b) the child has at least one other criterion for ANS dysregulation (Appendices, page 205), such as tachypnea for age, irregular or labored breathing but with oxygen saturation above 92% in air and a normal arterial blood gas, resting HR sustained above 150 beats/minute, mottled skin, profuse sweating, refractory fever, or hyperglycemia; and c) no contraindications to study inclusion are present, including a past history of hypertension, chronic renal, cardiac or pulmonary disease, or any neurological disorder; features indicating a current hypertensive emergency (see below); treatment with milrinone or any other inotropic agent has already commenced; respiratory distress is present with oxygen saturation below 92% in air or an arterial pCO2 over 45 mmHg; atrioventricular block or any arrhythmia (other than sinus tachycardia) is present on an ECG rhythm strip, or the QT interval is prolonged; or reduced urine output or increased creatinine levels indicating renal compromise. If the patient fulfilled these criteria and a parent or guardian gave written informed consent (see below), then the child was enrolled in the study.

Specific definitions for hypertension were as follows:

- For children aged 1 year and over, at least 3 consecutive SBP recordings above the 95th centile for age, gender and length (USA guidelines for defining Stage 1 hypertension in children [224], measured invasively over a period of 20 minutes provided the child is not distressed or crying.
- For children aged 6 months to 1 year, systolic BP > 100 mm Hg measured invasively on at least 3 occasions over a period for 20 minutes provided the child is not distressed or crying

The Vietnamese MoH guidelines specify that milrinone should be given to children

with HFMD and ANS dysregulation when the SBP is sustained at a level exceeding the 99th percentile for age plus 5 mmHg (i.e. Stage 2 hypertension) with the intention that treatment should commence within 1 to 2 hours [28]. Enrollment to the study was specifically designed to be early, when hypertension was first identified (at Stage 1), so that in the event of treatment failure, the MoH treatment guidelines could be applied. This stipulation was at the request of MoH, but it was agreed at the MoH's Institutional Review Board review that patients presenting with Stage 2 hypertension could also be enrolled provided all procedures were carried out rapidly and that if the SBP did not improve within 30 minutes of commencing the randomized study drug, milrinone would be added. Thus all patients with Stage 2 hypertension should be on milrinone within 1 hour of presentation unless the SBP had fallen to Stage 1 levels within this time frame.

4.2.3 Study endpoints

The primary endpoint was a composite endpoint indicating disease progression within 72 hours. It was defined as occurrence of any of the following within 72 hours of commencing the study drug infusion: a) specific BP criteria necessitating addition of milrinone as detailed below; b) need for mechanical ventilation; c) development of shock; and d) death.

The specific criteria for adding milrinone were:

- Hypertensive emergency – a severe symptomatic elevation in BP (> 30% compared to baseline BP) WITH evidence of acute target organ damage (e.g. brain, kidneys, eyes)
- SBP fails to decrease by 25% over the first 8 hours despite maximum MgSO4 maintenance infusion
- For children aged 1 year and over:
  - SBP increases to > 99th percentile plus between 5 -15 mm Hg consistently for 30 minutes
  - SBP increases to > 99th percentile plus 15 mm Hg consistently for 15 minutes

  - SBP increases to ≥ 40 mm Hg over baseline for 15 minutes, if this value is lower than either of the first two cut-offs. The baseline SBP is defined as the lowest value measured at any time after admission to hospital before enrolment in the study.
- For children aged 6 months to 1 years:
  - SBP increases to ≥110 mm Hg up to 120 mmHg consistently for 30 minutes
- SBP increases to ≥ 120 mm Hg consistently for 15 minutes

Secondary endpoints were originally planned to include comparison of the primary endpoint parameters singly, but finally the sample size was too small to allow this. Other secondary endpoints included the time to requirement for milrinone, and the Area Under The Curve (AUC) for HR, SBP, MAP above the Stage 1 hypertension level during the first 72 hours. In addition the duration of hospitalization, and neurodevelopmental status assessed 6 months after discharge using locally adapted and validated Bayley Scales of Infant and Toddler Development, Third Edition (Bayley-III) [225] were compared between the study arms.

Safety endpoints included the number of adverse events (AEs) and SAEs that occurred in the two treatment arms.

Exploratory endpoints: Comparisons of sequential daily plasma and urine catecholamine levels between the study arms. In addition, we also investigated correlations between catecholamine levels and clinical information including HR, SBP, and MAP during hospitalization.

4.2.4 Sample size calculation

During 2011 approximately one third of patients with Grade 3 HFMD managed at HTD (48/140, 34%) and CH1 (48/148, 32%) required milrinone for ANS dysregulation with hypertension, and in 9/48 (19%) of the HTD cases and 10/48 (21%) of the CH1 cases milrinone alone failed to control the hypertension. Following introduction of new Vietnam MoH management guidelines to commence intra-arterial BP monitoring early (at the first sign of autonomic disturbance), among 16 children managed in this way at

HTD over a 2 month period, 12/16 patients developed Stage 1 hypertension, and 7 of these 12 cases developed Stage 2 hypertension and were treated with milrinone. Based on this observation (i.e. that 7/12 children with Stage 1 hypertension progressed), we estimated a progression rate of 50% in the control arm for the sample size calculation for this study.

With respect to the hypothesized treatment effect there was little direct information for the actual scenario we planned to investigate, i.e. the influence of $MgSO_4$ commenced at Stage 1 hypertension on subsequent control of BP and progression to severe disease. In the case series described earlier, when $MgSO_4$ was added to the treatment regimen of patients with poorly controlled hypertension despite high dose milrinone, in all cases the BP reduced within 30-60 minutes and thereafter remained stable on a continuous $MgSO_4$ infusion. In the tetanus study mentioned previously (in which the study drug was given to patients with severe tetanus requiring a tracheostomy [171]) requirement for additional therapy to treat ANS dysregulation was reduced from 14/97 (14%) in the placebo group to 3/97 (3%) in the $MgSO_4$ group, although the need for assisted ventilation was similar in the two groups. Thus indirect evidence suggested that the effect size of the proposed intervention could be large; we therefore estimated that use of $MgSO_4$ could reduce the risk of progression by at >50%.

Based on 1:1 randomization, an anticipated relative reduction in the risk of progression of 50% (from 50% in the control arm to 25% in $MgSO_4$ recipients), 90% power and a two-sided 5% significance level, 85 patients per treatment group would be required. To allow for some violations of our assumptions and losses to follow-up, we planned to recruit 190 patients (95 patients per treatment arm) into the study.

4.2.5 Randomization and blinding

Randomization was carried out in a 1:1 ratio, with the intention to stratify according to the hospital where recruitment took place. A randomization list using block randomization with blocks of variable size was prepared using a computer program and maintained confidentially from study staff by the Clinical Trials Pharmacist. A

chronological log of all enrolled patients was maintained, with the next available sequential study code assigned to each patient as they enrolled. The assigned patient number corresponded to a coded, sealed, package containing 50 ampoules of MgSO4 or visually matched placebo.

4.2.6 Intervention

Following written informed consent a loading dose of 50mg/kg of either $MgSO_4$ or visually matched placebo was commenced by continuous infusion into a peripheral IV line over 20 minutes, followed by a maintenance infusion for 72 hours according to response, aiming for Mg levels 2-3 normal level in the treatment arm.

Patients were randomly assigned to one of the two treatment arms, and followed the same dosing schedule:

- *Group 1*: MgSO4 15% solution in sterile water in 10ml vials (Fresenius Kabi) diluted to 10% solution by mixing with 5 ml Nacl 0.9%
- *Group 2*: Sterile water in 10 ml vials (Fresenius Krabi) diluted to 15ml by mixing with 5 ml Nacl 0.9% .
- *Schedule:* Loading dose: 50mg/kg over 20 minutes (0.5ml/kg)
- Maintainance: 30–50 mg/kg/hr (0.3 ml/kg/hr to 0.5 ml/kg/hr) for 72 hrs

$MgSO_4$ and placebo were available in 10 ml visually matched ampoules supplied by Fresenius Kabi. Kits containing sufficient ampoules for each patient were prepared centrally by an unblinded study pharmacist to be distributed to the sites as required. All drugs were stored in accordance with the manufacturers' recommendations in a secure area, and all movements of study medication were recorded, with individual subject and overall drug accountability records kept by the study staff.

All staff involved in clinical care were blind to the treatment allocation. To maintain blinding Mg/Ca levels were monitored and adjusted by independent doctors from another clinical facility as detailed in the section on dose adjustment below.

4.2.7 Dose adjustment

After the initial loading dose the study infusion dose was increased in 0.1 ml/kg/hr stages (10mg/kg/hr) every 15 minutes to a maximum dose of 0.5 ml/kg/hr (50 mg/kg/hr), with the following caveats:

- If the SBP decreased to < 90th percentile for age, gender and length the dose should be reduced by 1 stage every 15 minutes
- If the SBP increased to the levels described below for treatment failure, action should be taken as indicated
- If the plasma Mg level was > 2.5 mmol/l or < 1.8 mmol/l a 25% increase or decrease in the infusion rate should be implemented as appropriate. To maintain blinding, plasma Mg/Ca values were sent to an independent doctor each morning who reviewed the results, followed a defined protocol and SOPs to decide on any changes, and then informed the PICU staff promptly of any dose adjustments to be made to the study drug infusion but did not report the actual lab values to the staff. Similar (sham) dose adjustments were made for the placebo infusions, according to a randomized list available only to the Mg/Ca monitoring doctor.

4.2.8 Recruitment of participants

Phase 1 (HTD only): Study staff working on PICU at HTD identified the parents/guardians of potentially eligible patients as soon as possible after admission. If the initial screening criteria were met, the staff provided the patient information sheet (PIS) to the family and discussed the study. Subsequently if a child developed autonomic disturbance with hypertension the study staff talked again to the family and requested consent, then followed the full pathway for inclusion/exclusion criteria, and if appropriate, proceeded to enrolment and randomization. Screening, enrolment, and obtaining informed consent were performed rapidly at this time, in order not to delay the commencement of necessary treatment.

In case worry over the child's illness had affected the parents/guardians ability to make an informed decision on study participation, a study doctor reviewed the PIS with the family at least once more 12-36 hours after enrolment.

Phase 2 (HTD and CH1): Initially it was planned that after enrolment of the first 30 patients the independent Data and Safety Monitoring (DSMB) would review all data, and unless specific safety concerns were identified recruitment would be expanded to include CHI. However actually no patients were recruited at CH1 before the trial closed.

4.2.9 Clinical assessments and laboratory measurements

Patients were examined daily by trained HDU/PICU study doctors. A record of all significant events in the previous 24 hours plus detailed physical examination findings (in particular the nervous system, respiratory and cardiovascular systems) was recorded each morning in the CRF. Vital signs, including HR, BP, respiratory rate, SpO2 were monitored at least hourly and recorded in a special nursing chart. A standard ECG recording was performed at the bedside by a trained nurse once daily, and also if any abnormality was noted on the monitor by study staff (eg. premature beats, atrial or ventricular arrhythmias). Urine output was monitored continuously using specific collecting bags and calculated every 4 hours.

All data were subsequently entered into an electronic database. Personal information was recorded on the CRF for the purpose of consent, and to enable follow-up of treatment outcomes, but access to identifying information was restricted to site-specific study staff and the clinical care team. Thus, only anonymised data was entered into the password-protected database. A system of double data entry was used to minimize data entry errors, as well as a data query and validation system to check all anomalies.

The following laboratory tests were performed on site in the standard hospital laboratories or at the OUCRU research laboratory.

- Blood glucose and arterial blood gases measured at least once daily or more frequently according to the clinical situation

- Plasma Mg and Ca concentrations measured at baseline and approximately 12 hours after the start of the study infusion, then once daily for the remaining 72 hours.
- Plasma electrolytes, creatinine, CK-MB and Troponin I measured at baseline and once daily thereafter.
- Plasma catecholamine (adrenaline and noradrenaline) concentrations measured at baseline and then once daily for 72 hours. Urine catecholamines measured every 24 hrs on an aliquot taken from the total urine collection since the previous day (BI-CAT ELISA Assay kit, Eagle Biosciences Inc.). (Urine was collected at the bedside into large bottles containing a small volume of 6M HCL, then aliquotted and frozen promptly, as described on page 222 of the Appendices)
- A nasal/throat swab and a rectal swab obtained at enrolment for enterovirus PCR, and serotype specific PCR if appropriate.

4.2.10 AEs and SAEs

Conventional definitions were applied for AEs and SAEs. All events were recorded on the patient CRF using specific forms, and were reported to the relevant authorities following specified protocols. In addition, for analysis of the safety endpoints the intensity of clinical and laboratory AEs was recorded on a five-point scale adapted from the NCI guidelines (CTCAE version 4.03)[226] to ensure that the cutoffs used were appropriate to the age of the children participating in the study (Appendices, page 212).

If any of the following occurred the study treatment was to be stopped immediately and rescue treatment given as appropriate (Appendices, page 228).

- Serious cardiac arrhythmia (eg atrio-ventricular block, prolonged QT interval)
- Hypotension: SBP < 70 + (2 X age) mmHg for 15 minutes or more
- Urine output < 1ml/kg/hr for 4 hours or more
- Cardiac arrest or any other emergency situation where the treating physician feels there is a contra-indication to the study drug.

Formal SOPs were written for all these scenarios, with detailed instructions for rescue treatment and for unblinding if necessary. In addition, if a patient developed respiratory distress (as defined in the Appendices, page 230) an urgent plasma Mg level was performed, with action to be taken following the relevant SOP.

The Clinical Trials Pharmacist maintained the unblinded randomization list with details of the contents of each individual treatment package. In the event of an AE where knowledge of the identity of the study treatment could contribute to the treating physician's ability to care for the patient, emergency unblinding could be authorized by any clinician involved in the patient's care, following the specified SOP and after discussion with me.

An independent DSMB was set up consisting of four Vietnamese and international experts with the necessary knowledge of paediatrics, clinical trials and statistics. The DSMB reviewed the protocol and agreed to a data review schedule and reporting requirements before the study commenced, with particular reference to SAE reporting. The Board reviewed the data after the first 5, and then the first 20 patients, and subsequently the members were consulted several times about the low recruitment rate and agreed to the final decision to terminate the study.

4.2.11 Follow up

All patients were assessed daily for the duration of the hospital stay. At discharge a full neurological and neurodevelopmental assessment was performed. However patients who had not recovered from the effects of sedation by the time of discharge but were otherwise considered fit to go home, were asked to attend one to two weeks later for formal review and neurological assessment. All patients were also asked to return at 6 months post-enrolment for a clinical and neurodevelopmental assessment. If any further follow-up was felt necessary after this, study participants were referred back to the standard hospital out-patient clinic system.

Neurodevelopmental assessments done at discharge (or 1-2 weeks later) and at 6 months used the Bayley-III and Movement ABC-2 tools – for children 36 months and under the Bayley-III was used, while for children aged 48 months and above the

Movement ABC-2 tool was used for the assessments. Children aged between 37 and 47 months at enrolment had both assessments done at both visits. The Bayley-III tool was recently translated and adapted for use in a Vietnamese population [225]. Data were collected on several hundred healthy Vietnamese children covering the appropriate age range, providing a suitable control population for comparison with the results from the severe HFMD cases.

5.2.10 Statistical analysis

The primary analysis population was defined as all randomized patients who commenced the loading dose infusion of study drug, with intention-to-treat analysis being done according to the randomized treatment arm. The per-protocol analysis that was intially planned was not performed due to the small size of the final study population. Similarly, the analysis that was initally planned in the subgroup of patients with confirmed EV-A71 infection was not conducted.

A formal data analysis plan was written before unblinding the data. As a general principle log-binomial regression, linear regression, and Cox regression were used respectively for binary endoints, continuous outcomes, and time-to-event endpoints, with the results diplayed as relative risk, mean difference, and hazard ratios, as appropriate. Log transformation was carried out if necessary for continuous outcomes with skewed distributions.

The primary endpoint of disease progression was compared between the two treatment arms based on univariate log-binomial regression, both unadjusted and after adjustment for age and day of illness at enrolment as covariates.

Among the secondary endpoints the time from commencement of study drug to addition of milrinone was analysed using univariate Cox regression with and without adjustment for baseline SBP and day of illness at study entry. Between-group comparisons of the AUCs for the various cardiovascular parameters assessed – HR, SBP and MAP above the Stage 1 level for each individual – were based on univariate linear regression and also linear regression with adjustment for the baseline value of the variable of interest. Length of hospital stay after study enrolment was also based on

univariate linear regression. For comparisons of the Bayley-III results, Z-scores for each domain were calculated by comparing the patient's score with the mean and standard deviation of the score for the same domain derived from a population of healthy children matched by age group (within 3 month age bands). These data came from a study of 250 healthy Vietnamese children from Ho Chi Minh City who were assessed using the Bayley-III in 2014-2015 [225]. Linear regression was used to calculate mean differences with and without adjusting for age, sex and maternal education level.

For the exploratory endpoints, plasma and urine catecholamine levels were compared between the treatment groups using linear mixed effects models, which modelled the mean time trend of the catecholamine levels as a linear function of the time from commencing the study drug adjusted for treatment allocation and including a random intercept. Correlations between each cardiovascular parameter (HR, SBP, and MAP) and each plasma or urine catecholamine level were assessed using Pearson correlations between residuals derived from linear regression models of these longitudinal measurements adjusted for treatment allocation and hours from drug administration. As cardiovascular parameters were measured more frequently than catecholamine levels, the residuals at all time-points for cardiovascular parameters were used, whereas for the catecholamine measurements, only the residuals at time-points which were closest to the time-points of the cardiovascular parameter measurements were used in the correlation analysis. Significance levels for these correlations were calculated using their Fisher transformation. Standard errors of these transformed correlations were derived using a cluster bootstrap technique, which resamples patients rather than samples, to take into account multiple measurements per patient.

4.3 Results

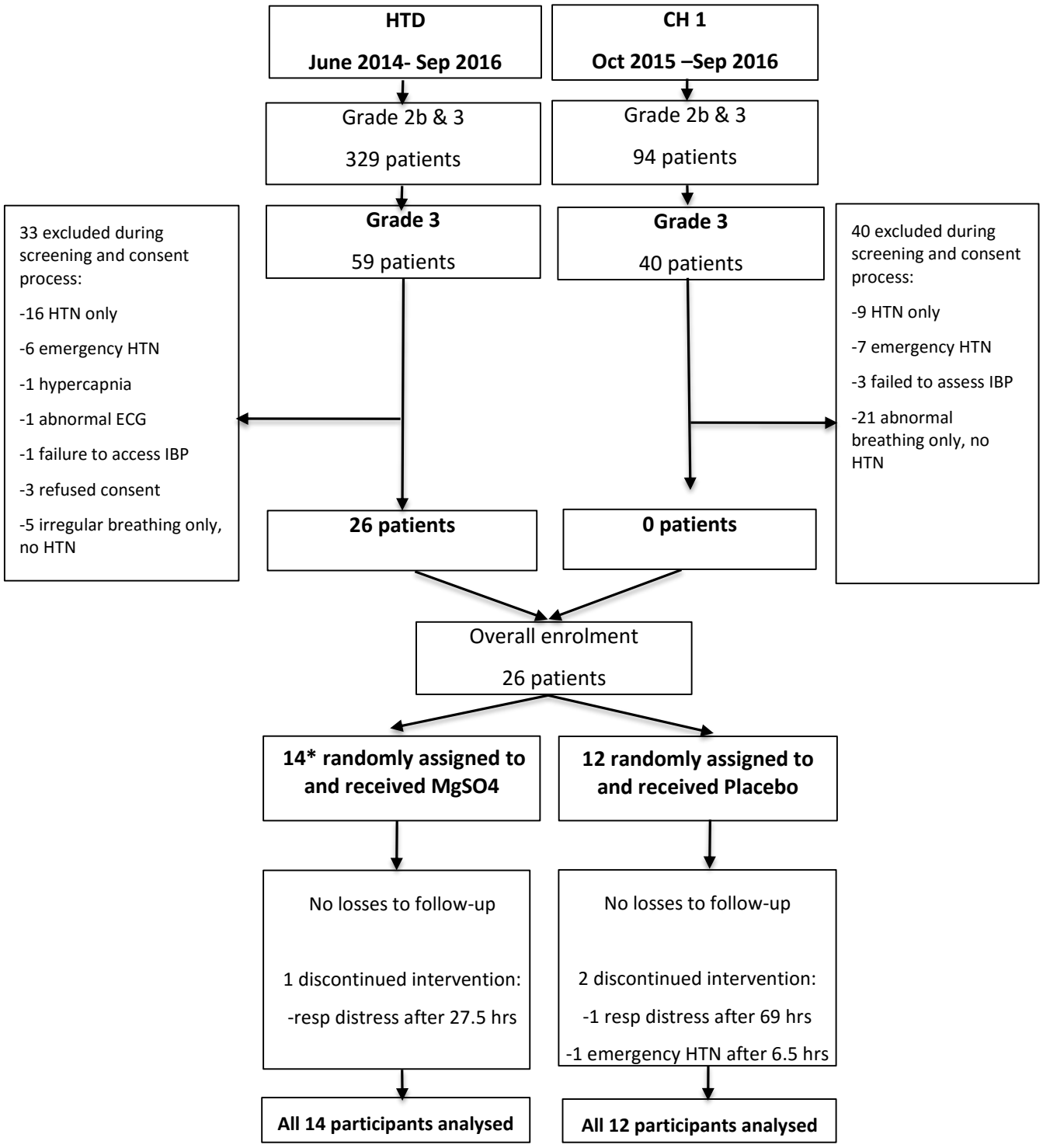
From June 2014 to September 2016, 329 HFMD cases with Grade 2b or above were admitted to the PICU at HTD. Among the 59 cases classified as Grade 3, 54 children developed hypertension and underwent full screening for potential inclusion in the study: 16 cases presented hypertension alone; 6 cases developed rapidly progressive hypertension that required milrinone immediately; 6 cases met various other exclusion criteria such as hypercapnia (1 case), abnormal ECG (1 case), failure to place arterial catheter (1 case); and 3 families declined consent for the study. Thus 26 children were screened and written consent was given by a parent or guardian, and these children were successfully enrolled in the study (Figure 4.1).

Following two reviews by the DSMB, after 5 and 20 cases had enrolled, the screening and enrolment process was extended to the CH1 site in October 2015. However by this time the HFMD numbers in the city were already declining. From that time onwards, 94 cases were screened at CHI, 40 of whom progressed to Grade 3 disease, 19 with hypertension. Unfortunately none of these children were successfully enrolled in the study however for the reasons indicated in Figure 4.1.

By the Spring of 2016 it was clear that the overall numbers of cases across Vietnam were much lower than previously. We considered trying to open a third site to boost recruitment numbers but felt that it would be very unlikely that we could achieve the required sample size within an acceptable time-frame even if many sites were opened. Thus, after consultation with the DSMB, recruitment was closed in October 2016, at which point a total of 26 cases were included in the study.

Note that after unblinding the Mg/Ca database kept by the independent doctors it became apparent that non-consecutive randomization had occurred for the last patient enrolled. This patient had been given ID Number 28 instead of 26, but all other processes were followed appropriately. The analysis was carried out according to the randomization code actually allotted.

Figure 4. 1: Patient recruitment and allocation algorithm



* For the last subject there was an error in the randomization process. Non-consecutive randomization occurred with 2 study numbers omitted inadvertently, such that the participant was allocated the treatment package for Participant 28 instead of Participant 26. All other procedures were followed correctly.

All children received the designated study drug according to the randomization code, and 23/26 cases completed the full 72 hour infusion as per the protocol. In one case emergency hypertension developed on Day 1, prompting the attending clinician to unblind the randomization; the child was in the placebo group and subsequently the doctor added open label MgSO4 to the regime, following which the BP settled without additional therapy. Two other cases experienced SAEs (respiratory distress) requiring ventilation, and the treatment code was unblinded for safety reasons. One child was in the placebo group and the Mg/Ca levels were within the normal range. The other child was in the active intervention arm, and in this case the plasma Mg level at the time of unblinding was 2.3mmol/l with a Ca level of 1.9mmol/l. The study drug was stopped, milrinone was added to control the BP, and the child was ventilated. He recovered fully and was discharged home on day 14.

There were no protocol violations, and a total 11 protocol deviations, but all were minor.

4.3.1 Summary of baseline features at study enrolment

The baseline characteristics at randomization were similar between the MgSO4 and placebo groups (Table 4.1). All participants were admitted and enrolled into the study at a median of illness day 3. The median (range) age in the MgSO4 group was slightly greater than in the placebo group [24.4 (8.5, 72.7) vs. 21.1 (7.3, 57.1)] months, but the difference was small; 9/14 (64%) cases in the MgSO4 group were female compared to 6/12 (50%) cases in the placebo group. Weight and length were similar between the two study groups.

Clinical features were also quite similar in terms of fever, skin and mouth lesions, and also general cardiovascular and respiratory parameters. Skin ANS features rarely occurred, with no children noted to have profuse sweating and only 3 cases with mottled skin. The median HR, SBP and diastolic blood pressure (DBP) were marginally higher in the placebo group than in the MgSO4 group, but when considered in the light of the internationally accepted staging of hypertension the proportion with Stage 2

Table 4.1: Baseline demographic information and clinical features

	All patients (N=26)	Placebo (N=12)	MgSO4 (N=14)
Illness day at enrolment	3.0 (1.0, 6.0)	3.5 (1.0, 6.0)	3.0 (2.0, 5.0)
Age (months)	23.1 (7.3, 72.7)	21.1 (7.3, 57.1)	24.4 (8.5, 72.7)
Sex (female)	15 (58)	6 (50)	9 (64)
Length (cm)	86 (66, 108)	86 (66, 108)	85 (66, 107)
Weight (kg)	10.7 (6.6, 20.0)	11.0 (6.8, 19.3)	10.7 (6.6, 20.0)
Temperature (^{0}C)	38.5 (37.5, 40.0)	38.5 (38.0, 39.5)	38.5 (37.5, 40)
Mouth ulcers present	21 (81)	10 (83)	11 (79)
Skin lesions present	20 (77)	9 (75)	11 (79)
Mottled skin	3 (12)	2 (17)	1 (7)
Abnormal CRT (> 2s)	1 (4)	1 (8)	0 (0)
Liver palpable	8 (31)	5 (42)	3 (21)
HR (beats/min)	150 (122, 170)	153 (122, 170)	150 (130, 170)
Tachycardia*	8 (31)	2 (17)	6 (43)
SBP (mmHg)	113 (101, 134)	116 (105, 129)	113 (101, 134)
Stage of hypertension			
- Stage1	15 (58)	4 (33)	11 (79)
- Stage2	11 (42)	8 (67)	3 (21)
DBP (mmHg)	61 (47, 73)	61 (52, 73)	59 (47, 73)
SpO2 (%)	99 (94, 100)	99 (96, 100)	99 (94, 100)
Respiratory rate (/min)	37 (23, 70)	39 (24, 70)	36 (23, 55)
Irregular breathing	12 (46)	7 (58)	5 (36)
Respiratory retraction	4 (15)	1 (8)	3 (21)
Irritable	5 (19)	4 (33)	1 (7)
Witnessed myoclonic	2 (8)	2 (17)	0 (0)
Limb tremor / ataxia	1 (4)	1 (8)	0 (0)
Nystagmus	1 (4)	1 (8)	0 (0)

Summary statistic is absolute count (%) for categorical variables and median (range) for continuous data

CRT: Capillary Refill Time; SBP: Systolic blood pressure, DBP: Diastolic blood pressure

*: Heart rate sustained >150 beats/min, adjusted down by 10 for each 1 degree of fever above 37.0 oC

hypertension was considerably higher in the placebo group (8/12, 67%) than in the MgSO4 group (3/14, 21%). The median respiratory rate and proportion with irregular breathing were also marginally higher in the placebo group than in the MgSO4 group, but the proportion with respiratory retractions was lower. The SpO2 in air was much the same in both groups.

There were no cases in either group with altered consciousness, assessed in term of the modified Glasgow Coma Score for young children, but 4/12 (33%) of the participants in the placebo group were considered to be irritable at enrolment as compared to 1/14 (7%) in the MgSO4 group. Other neurological manifestations including ataxia and nystagmus were rare, only occurring in one patient each (different cases) in the placebo group. Witnessed myoclonic jerks were rather infrequent but this likely reflects the fact that all patients with severe HFMD were routinely sedated on admission.

The baseline laboratory results are shown in Table 4.2. Median (range) plasma Mg levels were quite similar at baseline in the MgSO4 group [0.88 (0.78, 0.98)] mmol/l] compared to the placebo group [0.85 (0.70, 0.99)] mmol/l. However 5 cases in the placebo group had values below the local normal range (0.8-1.0 mmol/l) while only 1 case in the MgSO4 group had a low level. There were no values in either study arm that were above the normal range at baseline.

Some abnormalities were noted in various baseline tests but these were well distributed between the groups. Thus hyponatremia was quite common, and also mild to moderate leukocytosis. One child in the MgSO4 group had a very low Hb at enrolment, 6.4 g/dL, with a blood picture consistent with iron deficiency. Serum iron and ferritin were very low and the child commenced oral iron supplements which were continued at discharge. The anemia was considered to be of nutritional origin.

Table 4.2: Baseline laboratory investigations

	All patients (N=26)	Placebo (N=12)	MgSO4 (N=14)
Arterial blood gases			
- pH	7.42 (7.36, 7.59)	7.44 (7.40, 7.59)	7.41 (7.36, 7.48)
- pO2 (mmHg)	107 (75, 161)	104 (75, 144)	123 (80, 161)
- pCO2 (mmHg)	31.8 (18.6, 40.1)	29.8 (18.6, 35.7)	33.3 (29.4, 40.1)
- HCO3 (mmol/l)	21.3 (17.8, 25.5)	20.8 (17.8, 23.2)	22.1 (19.2, 25.5)
Hb (g/dl)	11.8 (6.4, 15.2)	11.8 (8.8, 15.2)	11.8 (6.4, 14.3)
WBC ($x10^9$/L)*	12.2 (4.6, 26.5)	10.2 (4.6, 15.5)	13.6 (5.5, 26.5)
NEU ($x10^9$/L)	5.9 (2.1, 15.1)	5.6 (2.1, 10.6)	6.6 (2.4, 15.1)
LYM ($x10^9$/L)	3.2 (1.0, 11.2)	3.1 (1.6, 5.5)	4.3 (1.0, 11.2)
PLT ($x10^9$/L)	340 (172, 641)	289 (196, 462)	384 (172, 641)
CRP (mg/l)	1 (0, 167)	2(0, 167)	1 (0, 45)
CK-MB (UI/l)**	24.9 (10.4, 64.8)	23.4 (10.4, 64.8)	25.45 (13.8, 48.1)
Troponin I (pg/ml)	5.3 (0.0, 27.0)	5.25 (0.0, 20.3)	5.35 (0.0, 27.0)
Mg (mmol/l)***	0.87 (0.70, 0.99)	0.85 (0.70, 0.99)	0.88 (0.78, 0.98)
Ca (mmol/l) ***	2.28 (1.69, 2.52)	2.28 (1.69, 2.52)	2.31 (1.95, 2.42)
Na (mmol/l)	131 (125, 136)	131 (126, 134)	131 (125, 136)
K (mmol/l)	3.88 (3.10, 4.59)	4.01 (3.10, 4.59)	3.67 (3.42, 4.34)
Creatinine (μmol/l)	26.5 (17.0, 52.0)	26.0 (17.0, 52.0)	26.5 (18.0, 42.2)
Glycemia (mmol/l)	5.7 (4.0, 8.1)	5.4 (4.0, 7.6)	5.7 (5.0, 8.1)

Summary statistic is median (range) for all variables

*: FBCs performed within the 24 hours prior to enrolment were not repeated at study enrollment.

**: CK-MB was not done in one case because the reagent was out of stock on that day

***: Blood for Mg/Ca levels was taken 15-45 minutes after commencing the drug in three cases in the MgSO4 group

Viral diagnostic results between the two treatment arms

Results of the viral diagnostic testing showed 18/26 (70%) of cases to be positive for an enterovirus (Table 4.3). Among these cases, EV-A71 was dominant with 9/14 (64%) confirmed cases in the MgSO4 group and 4/12 (33%) in the placebo group. Other enterovirus serotypes, including CV-A16, CV-A10 and EV-C, were also identified but

only in small numbers. In total in 5/12 (42%) in the placebo group and 3/14 (21%) in the MgSO4 group the enterovirus PCR was negative.

Table 4.3: Enterovirus serotypes identified among patients in the two treatment arms

Enterovirus serotype	All patients (N=26)	Placebo (N=12)	MgSO4 (N=14)
EV-A71	13 (50)	4 (33)	9 (64)
CV-A16	3 (12)	1 (8)	2 (14)
CV-A10	1 (4)	1 (8)	0 (0.00)
EV-C	1 (4)	1 (8)	0 (0.00)
Negative	8 (30)	5 (42)	3 (21)

Summary statistic is absolute count (%) for all variables

EV-A71: enterovirus A71, CV-A16 and CV-A10: Coxsackievirus A16 and A 10, EV-C: enterovirus group C

4.3.2 Relationships between Mg and Ca levels in the two treatment arms.

All patients had samples checked at baseline (Day 0) and between 8-10 am every morning for 3 days. In addition study participants admitted in the afternoon had a sample taken between 8-10pm on the first day (referred to as mid-point values), primarily to ensure high levels with potential toxicity were not being missed until the next morning. Since patients were enrolled at different times of the day, the interval between the baseline and the mid-point value was variable, but intervals between subsequent daily samples were all approximately 24 hours. For the final sample on the morning of Day 3, most individuals had already completed the 72-hour study schedule and stopped the study drug by this time; thus only 3 cases had notably increased levels at this time (Figure 4.2).

As expected in the MgSO4 group, the level of Mg increased in plasma during the period of the infusion and then returned to normal after stopping the drug. While in the placebo group the Mg/Ca levels remained stable for the duration of the study. A clear relationship was observed between the serum Mg and Ca levels as shown in Figure 4.2. The median Ca level in the MgSO4 group decreased from around 2.28 mmol/l at baseline to a minimum of 1.85 mmol/l on Day 1 then increased again to 2.13

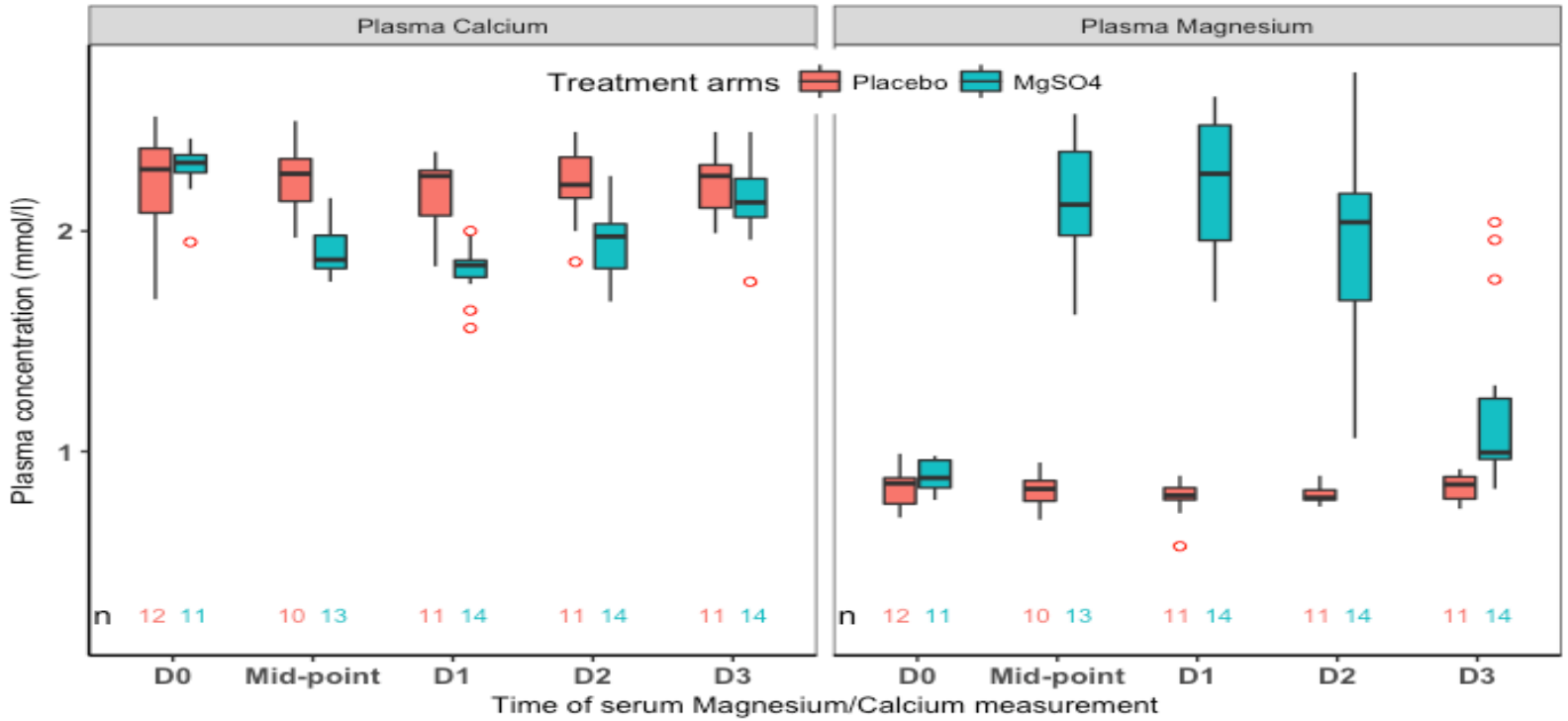
mmol/l on Day 3. A Ca level of 1.8 mmol/l had been designated as the level at which the independent doctor would recommend giving a bolus of Calcium; however in no patient in the MgSO4 arm was this needed.

From the safety perspective, the Mg level did not exceed 3 mmol/l (the stopping criterion in the SOP) in any child, with the maximum recorded value begin 2.72 mmol/l. A total of 7/130 (5%) measurements taken in the MgSO4 group were in the 2.5-3 mmol/l range, resulting in the independent monitoring doctor recommending to the attending clinician a dose reduction in 6 cases. In the 7th case the independent doctor did not act on a value of 2.52 mmol/l obtained at the mid-point of Day 1 and only marginally above the value of 2.5 mmol/l specified in the SOP for dose reduction; the subsequent measurement in the same patient the following morning was 2.29 mmol/l without any change to the infusion rate.

An error occurred when two mid-point patient samples were sent to the laboratory at the same time and the technician mixed the samples up. Based on an apparent result of 0.86 mmol/l instead of 2.24 mmol/l, the independent doctor recommended that the clinical doctor increase the study drug infusion but this was not done since the infusion was already at the maximum permitted rate, i.e 0.5 ml/kg/hr. The next level was 2.53 mmol/l, following which the infusion rate was appropriately reduced on the advice of the independent doctor. Conversely, the “wrong” level in the other (placebo) case (i.e. 2.24 mmol) was within the safe range, and so no change was recommended. In none of these cases did any AEs occur.

A total of 22/130 (17%) of the plasma values in the MgSO4 group were below the estimated lower limit for a therapeutic effect in this study (1.8 mmol/l) at some point during the 72 hours; 3 measurements at the mid-point of Day 0, 1 on Day 1, 6 on Day 2, and 12 on Day 3. The early low values responded to an increase in the infusion rate, while many of the late low values were because the patients had stopped the drug infusion (one unblinded following an SAE, and another 6 who had already completed their 72 hour infusion by the morning of Day 3).

Figure 4.2: Boxplots showing the Magnesium and Calcium levels in the two treatment arms.



D0 = baseline values, Mid-point = values measured 8-16 hrs after commencing the study drug. Other values (D1, D2, D3) were measured at 8-10 am on the relevant study day. The number or study participants with values at each time point are indicated along the bottom of the graph.

Note: Mg/Ca results were excluded for the following reasons. A) One placebo case was unblinded and commenced open label MgSO4. B) Two mid-point samples from different children were mixed up in the biochemistry lab. C) Three children in the MgSO4 arm did not have their baseline samples taken until 15-45 minutes after commencing the study drug infusion.

4.3.3 Comparison of the primary and secondary endpoints between the two treatment arms.

Effect of MgSO4 on the composite primary outcome (Table 4.4).

No patient died or developed shock in either study arm. A total of 6/12 (50%) cases required addition of milrinone in the placebo group compared to 6/14 (43%) cases in the MgSO4 group, while 2 cases in total required ventilation, one child in each group. These two children also required milrinone. Thus the analysis of the composite endpoint (including these four parameters), showed that the proportion who experienced the primary endpoint was very slightly higher in the placebo group (6/12, 50%) than in MgSO4 group (6/14, 43%), but there was no significant difference between the two groups with a relative risk (95%CI) of 0.86 (0.37, 1.96), p=0.715. When adjusting for age and illness day the result was very similar, with a relative risk (95%CI) of 0.96 (0.43, 2.16), p=0.923.

Effect of MgSO4 on hemodynamic parameters and duration of stay (Table 4.4)

Evaluation of the AUCs for the various cardiovascular parameters assessed – HR, SBP and MAP above the Stage 1 level for each individual patient – showed each AUC to be slightly lower in the MgSO4 group compared to the placebo group, but there were no statistically significant differences using linear regression (p= 0.168, 0.562 and 0.468 respectively). Repeat analysis including adjustment for the respective baseline values for these parameters also showed no differences.

The duration of hospital stay did not differ between the treatment arms.

Table 4.4: Comparison of primary and secondary endpoints between the two treatment arms

Characteristic	Placebo (N=12)	MgSO4 (N=14)	Estimated effect [&]	p-value	Estimated effect after adjustment*	p-value
Primary endpoint [#]	6 (50)	6 (43)	0.86 (0.37, 1.96)		0.96 (0.43, 2.16)	0.923
Required milrinone	6 (50)	6 (43)				
Required ventilation	1 (8)	1 (7)				
Elapsed time to start of milrinone (hours)	45.8 (0.6, 72.0)	72.0 (1.2, 72.0)				
Duration of hospitalization (days from enrolment)			0.12 (-1.55, 1.79)	0.889		
- 4	3 (25)	3 (21)				
- 5	4 (33)	5 (36)				
- 6	3 (25)	5 (36)				
- 9	2 (17)	0 (0)				
- 14	0 (0)	1 (7)				
AUC of HR (beats/min x hr)	9558 (8940, 10334)	9018 (8625, 9560)	-456 (-1103, 191)	0.168	-529 (-1237, 180)	0.144
AUC of SBP above stage 1 HTN (mmHg x hr)	305 (190, 469)	253 (105, 458)	-83 (-364, 198)	0.562	-53.82 (-327, 219)	0.699
AUC of MAP above stage 1 HTN (mmHg x hr)	162 (53, 282)	144 (94, 212)	-89 (-277, 99)	0.468	-52.90 (-242, 137)	0.584

Summary statistic is absolute count (%) for categorical variables and median (range) for continuous data

#: composite outcome including death, shock, requirement for milrinone and ventilation.

&: Relative risk, mean difference based on the Log-binomial regression, linear regression for binary outcome, continuous outcome, respectively.

*: Primary endpoint was adjusted for age and illness day; AUCs of HR, SBP and MAP were adjusted for their respective values at baseline.

Comparison of elapsed time from baseline to addition of milrinone (Table 4.5, Figure 4.3)

Overall 12 patients (6 in each group) required addition of milrinone because the study drug failed to control hypertension, in line with the predefined criteria. The median time from commencing the study drug to addition of milrinone was longer in the MgSO4 group compared to the placebo group, with a median (IQR) of NA (2.25, NA) hours vs. 45.75 (1.62, NA) hours respectively (NA-Not available). Using Cox regression the difference was not statistically significant, with a hazard ratio (95% CI) of 0.80 (0.26, 2.47); p=0.694 (Table 4.5). In the Kaplan-Meier analysis, there was no significant difference in the milrinone-free trajectories between the treatment groups (logrank test, p=0.702) (Figure 4.3).

Table 4.5: Comparison of the time elapsed from baseline to commencement of milrinone between the two study arms, using Cox regression analysis with and without adjustment for baseline SBP and illness day.

	Placebo (n=12)	MgSO4 (n=14)	Before adjusting		Adjusted*	
			Comparison	p-value**	Comparison	p-value**
	events/n (risk [%])	events/n (risk [%])	HR (95%CI); p-value		HR (95%CI); p-value	
Milrinone use	6/12 (50)	6/14 (43)	0.80 (0.26, 2.47); p=0.694	0.564	0.80 (0.25, 2.55); p=0.706	0.486
Time to addition of milrinone (hours)						
-Median (IQR)	45.75 (1.62, NA)	NA (2.25, NA)				

Summary statistic is absolute count (%) for categorical variables and median (IQR) for continuous data; HR =Hazard Ratio

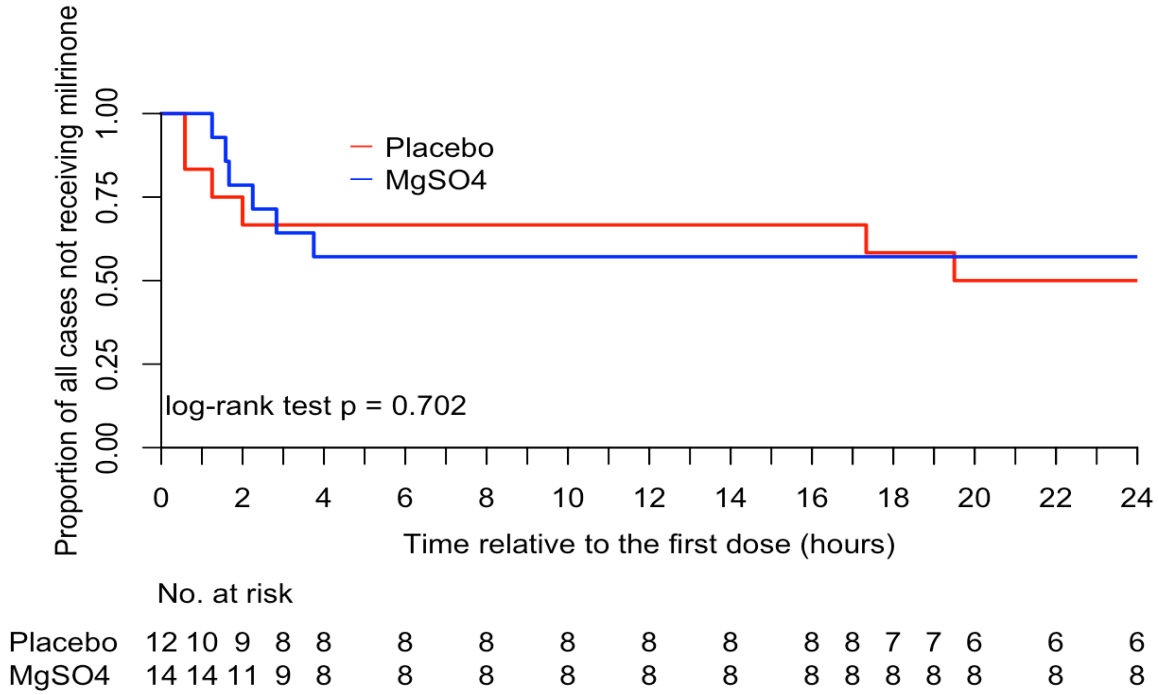
The upper IQR of time to milrinone addition (i.e. the time at which 75% patients received milrinone) in the placebo group was unidentified as only 50% of patients in this group received milrinone.

The median and upper IQR of time to milrinone addition (i.e. the time at which 50% & 75% patients received milrinone) in the MgSO4 group were unidentified as only 43% of patients in this group received milrinone.

*: Adjusted for SBP at baseline and illness day

**: test for proportional hazards

Figure 4.3: Kaplan-Meier curve showing the time in hours from baseline to addition of milrinone in the two study arms.



4.3.4 Comparison of neurological and neurodevelopmental assessments at discharge and 6 months later between the two treatment arms

All study participants had recovered sufficiently to be discharged home by Day 14 after enrolment. The duration of hospital stay did not differ between the treatment arms. At discharge 3 children had clinically apparent neurological problems, while the remaining 23 patients had no detectable neurological abnormalities on examination.

A 72 month old child (MgSO4 treatment arm) who had required high dose milrinone for 5 days and developed respiratory distress needing ventilation for 6 days, also complicated by a secondary pneumonia, had generalized weakness involving all four limbs when discharged. At an interim assessment 3 months later he had recovered completely. A 14 month old girl (placebo group) had severe hypertension requiring nicardipine after failing on high dose milrinone, developed respiratory distress and was ventilated for 3 days, and also experienced profound hyponatraemia with a Na level of 118 mmol/l on study day 3 that improved gradually to 135 mmol/l by study day 6. At discharge she had 9^{th} and 11^{th} cranial nerve palsies, which were still present but improved by 3 months, but had resolved by the formal 6-month visit. The third child, also in the placebo group, was aged 29 months; he did not have a particularly complicated clinical course and did not require milrinone, but at discharge he presented generalized weakness of all four limbs. He was reviewed one month later and had made a full recovery by that time.

For children aged 36 months or younger at enrolment, neurodevelopmental assessments were performed by trained examiners using the Vietnam adapted Bayley-III tool. No children were within the age bracket 36-48 months, while 2 children (one in each group) were older than 48 months and were tested using the Movement ABC-2 tool. For these two children, aged 57 and 73 months, the findings were abnormal in one case at discharge and totally normal at 6-month assessment, but no specific data will be presented here.

Among the remaining 24 cases, one child was of ethnic Chinese origin and was too young to understand instructions from the Vietnamese examiner, one 14 month old child would not cooperate at the first assessment two weeks after discharge but did

complete the assessment after 6 months, while another child did complete the early assessment but would not cooperate with the 6-month assessment. Therefore, the data presented here refer to assessments on 22 children.

Z-scores, normalised to the control population of healthy Vietnamese children previously assessed by Dr Sabanathan [225] were compared between the two treatment arms (Table 4.6). Minor differences were observed in all five domains at discharge, but the only area in which a significant difference was demonstrated was in the domain relating to expressive communication, where the Z-scores were slightly higher in the MgSO4 group compared to the placebo group, p=0.022 in the unadjusted analysis, and p=0.031 after adjustment for potential confounders including sex, age and maternal education level.

All 26 children attended the 6-month follow up visit, and neurological examination was normal in all cases. For the neurodevelopmental assessments at 6 months the Z-scores for the 5 domains were consistently higher than those at discharge, but there were no differences between the treatment arms at this time. In-hospital sedation may have contributed to the slightly lower Z-scores at discharge, but by 6 months the neurodevelopmental status of children in both groups was comparable with that of their peers who had not experienced severe HFMD. On this analysis of a small number of cases, no adverse effects of MgSO4 were demonstrated after 6 months.

Table 4.6: Comparison of Bayley-III neurodevelopmental assessments at discharge and 6 months later between the two treatment arms

	n [#]	Placebo (N=12)	n	MgSO4 (N=14)	Unadjusted mean difference	p-value	Adjusted mean difference*	p-value
At discharge								
CS	9	0.1 (-1.3, 1.2)	13	-0.3 (-2.3, 2.5)	-0.32(-1.30, 0.65)	0.474	-0.42 (-1.56, 0.72)	0.365
RC	9	-0.6 (-1.5, 1.1)	13	-0.0 (-1.3, 1.2)	0.39 (-0.31, 1.10)	0.229	0.38 (-0.45, 1.20)	0.265
EC	9	-0.3 (-2.1, 1.3)	13	0.3 (-1.2, 1.5)	0.98 (0.10, 1.87)	**0.022**	0.90 (-0.08, 1.88)	**0.031**
FM	9	-0.1 (-0.9, 1.0)	13	-0.4 (-1.2, 2.3)	0.11 (-0.76, 0.98)	0.785	-0.06 (-0.97, 0.86)	0.876
GM	9	-0.1 (-1.2, 0.7)	13	0.3 (-2.0, 1.5)	0.26 (-0.61, 1.13)	0.521	0.33 (-0.66, 1.32)	0.409
Six months after discharge								
CS	10	2.5 (0.1, 5.5)	12	1.4 (-1.3, 4.1)	-0.73 (-2.11, 0.65)	0.254	-0.28 (-1.38, 0.83)	0.532
RC	10	1.1 (-5.3, 8.8)	12	1.5 (-0.6, 5.2)	0.26 (-2.12, 2.63)	0.813	0.61 (-1.40, 2.61)	0.455
EC	10	1.2 (-1.0, 3.0)	12	1.6 (-1.0, 5.1)	0.18 (-1.13, 1.49)	0.765	0.61 (-0.65, 1.86)	0.238
FM	10	2.6 (0.2, 5.5)	12	1.9 (-0.6, 3.5)	-0.92 (-2.18, 0.34)	0.876	-0.79 (-2.12, 0.54)	0.150
GM	10	1.9 (0.0, 4.0)	12	2.2 (0.4, 3.8)	0.07 (-0.91, 1.05)	0.876	0.37 (-0.59, 1.33)	0.341

Summary statistic is the median (range) of Z-scores

#: 2 children older than 48 months were assessed by Movement ABC-2 tool; 1 child could not be assessed as the mother tongue was Chinese, 1 child would not cooperate with the test at discharge and 1 child (a different case) at the 6 month visit.

*: Mean differences of Z-scores were adjusted for sex, age, and maternal education level

CS= Cognitive Scale; RC: Receptive Communication; EC= Expressive Communication; FM= Fine Motor; GM= Gross Motor

4.3.5 AEs and SAEs in the two treatment arms

AEs are presented in Table 4.7, including both the total number of AEs in each group, and also the total number of patients with any AE. A total of 21 events occurred in 10 patients in the placebo group (83%) compared to 22 events in 10 children in the MgSO4 group (71%). Fever related AEs were the most common in both groups, followed by reduced urine output to less than 1 ml/kg/hr for consecutive 4 hours, but all these cases resolved with observation and/or standard supportive care only. Depressed tendon reflexes were also relatively common, but in all cases the children were receiving parenteral sedation in accordance with MoH guidelines.

Respiratory events (irregular breathing, retractions, stridor) occurred in 2 patients in the placebo group and 3 in the MgSO4 group. Among these cases, 2 developed severe respiratory distress requiring ventilation and were classified as Grade 4 SAEs (one patient in each group). An additional patient in the MgSO4 group required increased oxygen but in the end did not need respiratory support and this was finally classified as a Grade 3 SAE. In the placebo group the only other SAE reported (Grade 3) was in the patient who required ventilation for severe respiratory distress; this child had impaired consciousness with a GCS reduced to 9 the day before she was ventilated.

In accordance with the relevant SOPs Mg/Ca levels were checked in response to the various clinical scenarios. There were no cases in which the AEs or SAEs were associated with levels of Mg that exceeded 3 mmol/l, the generally accepted threshold at which respiratory or neurological events may occur.

Table 4.7: Clinical AEs and SAEs in the two groups

	Placebo (n=12)	MgSO4 (n=14)
Any AE*	21 / 10 (83)	22 / 10 (71)
Fever	6	7
Diminished deep tendon reflexes	2	3
Coma / lethargy / irritability	2	1
Respiratory problems	3	4
Reduced urine output (<1ml/kg/hr for 4 hrs)	4	5
Other**	4	2
Any SAE (Grades 3 and 4)*	3 / 2 (17)	3 / 3 (21)
Respiratory distress	2	3
Coma	1	0

*: Numbers are total events / events grouped by patient (% of patients in the group with any event)

**: Placebo group: 2 case each of red skin, diarrhoea, measles

**: MgSO4 group: 1 case of vomiting, myoclonic jerk

Definitions for the various laboratory abnormalities, adapted from the US CTCAE guidelines are described in the Appendices (page 212). Table 4.8 presents all laboratory AEs that occurred after enrolment, with those classified as Grade 3 or 4 also listed separately. Equivalent numbers of AEs and SAEs occurred in the two treatment arms. A total of 32 AEs occurred in 12 (100%) of the placebo group compared to 29 AEs in 12 (86%) of the MgSO4 group. The most common AEs, observed at similar rates in both groups, were respiratory alkalosis and hyponatremia. Many children were tachypnoeic thus explaining the respiratory alkalosis; in a number of them the abnormalities fulfilled the CTCAE criteria for an SAE, but in all cases the patients clinical condition improved without intervention. Sodium levels were commonly below 130 mmol/l, with values this low noted in 5/12 (42%) patients in the placebo group and 7/14 (50%) cases in the MgSO4 group. None of these patients were receiving IV fluids other than for drug delivery. However in one child the sodium fell to 118 mmol/l on study day 3, by which time the child was receiving IV fluid (Ringer Lactate) at 5 ml/kg/hour while being ventilated.

Minor creatinine abnormalities were noted occasionally (Grade 1 only) and no serious renal problems were encountered in either treatment group. Abnormal cardiac enzymes (CK-MB and Troponin I) also occurred with similar frequency in both groups, and in some cases the Troponin I values qualified as Grade 3 SAEs. However none of the patients had clinical evidence of cardiac involvement and all the ECGs were within normal limits including the daily evaluation of the QT interval.

Table 4.8: Laboratory AEs and SAEs in the two treatment arms (new after enrolment)

	Placebo (n=12)	MgSO4 (n=14)
All AEs*	32 / 12 (100)	29 / 12 (86)
Respiratory Acidosis	0	2
Respiratory Alkalosis	8	4
Abnormal CK-MB	5	6
Abnormal Creatinine	2	1
Abnormal Hb	0	1
Hyperkalemia	2	0
Hypokalemia	4	2
Hyponatremia	7	8
Abnormal Troponin I	4	5
SAEs (Grade 3-4)*	14 / 8 (67)	17 / 9 (64)
Respiratory Acidosis	0	1
Respiratory Alkalosis	3	4
Abnormal CK-MB	1	0
Hypokalemia	1	1
Hyponatremia	5	7
Abnormal Troponin I	4	4

*: Numbers are total events / events grouped by patient (% of patients in the group with any event)

4.3.6 Effects of MgSO4 on catecholamine levels (Table 4.9 and Figure 4.4)

Plasma catecholamine levels, specifically adrenaline and noradrenaline, were measured on specimens collected at enrolment and then subsequently every morning between 8-10am for the next 3 days. The catecholamine levels in plasma and urine were generally elevated or at the upper end of the of the expected normal ranges for children (Figure 4.4).

Although at enrolment median plasma levels of adrenaline were lower in the MgSO4 group than in in the placebo group, median (range) 92.5 (20.0, 370.0) vs. 137.5 (0.0, 590.0) pg/ml in the two groups respectively, over the next few days as the levels were falling in both groups, relationships between the two study arms varied. Using a linear mixed effects regression model that accounted for the evolution of the responses in the two groups, no significant difference was found in the overall profiles between the study arms (p=0.609). Also the regression analysis did not show a statistically significant reduction over time in the adrenaline concentrations (p=0.284). A similar pattern was seen in the plasma noradrenaline levels – i.e. an apparent reduction over time that was variable on the individual day measurements between the two study arms, but no significant findings overall either for the comparison between the study arms or for the evolution over time (p=0.997 and p=0.466, respectively).

No urine was obtained at baseline, but all urine was collected from that time onwards until 8am the following morning, with ongoing 24-hour collections thereafter for the 72-hour study period. Similar to the plasma findings, urine levels of adrenaline and noradrenaline were slightly lower in the MgSO4 group than in the placebo group during the first 24-hour period (Day1) but relationships between the two study arms were variable subsequently. Using linear mixed effect regression models, no significant differences were detected between the study arms. However with respect to the evolution of urine catecholamine concentrations over time, both adrenaline and noradrenaline were confirmed to show a progressive decline (p= 0.032 and p=0.013, respectively).

Table 4.9: Comparison of catecholamine levels in plasma and urine between the two study arms, by fixed effect regression adjusting for the time and day of assessment

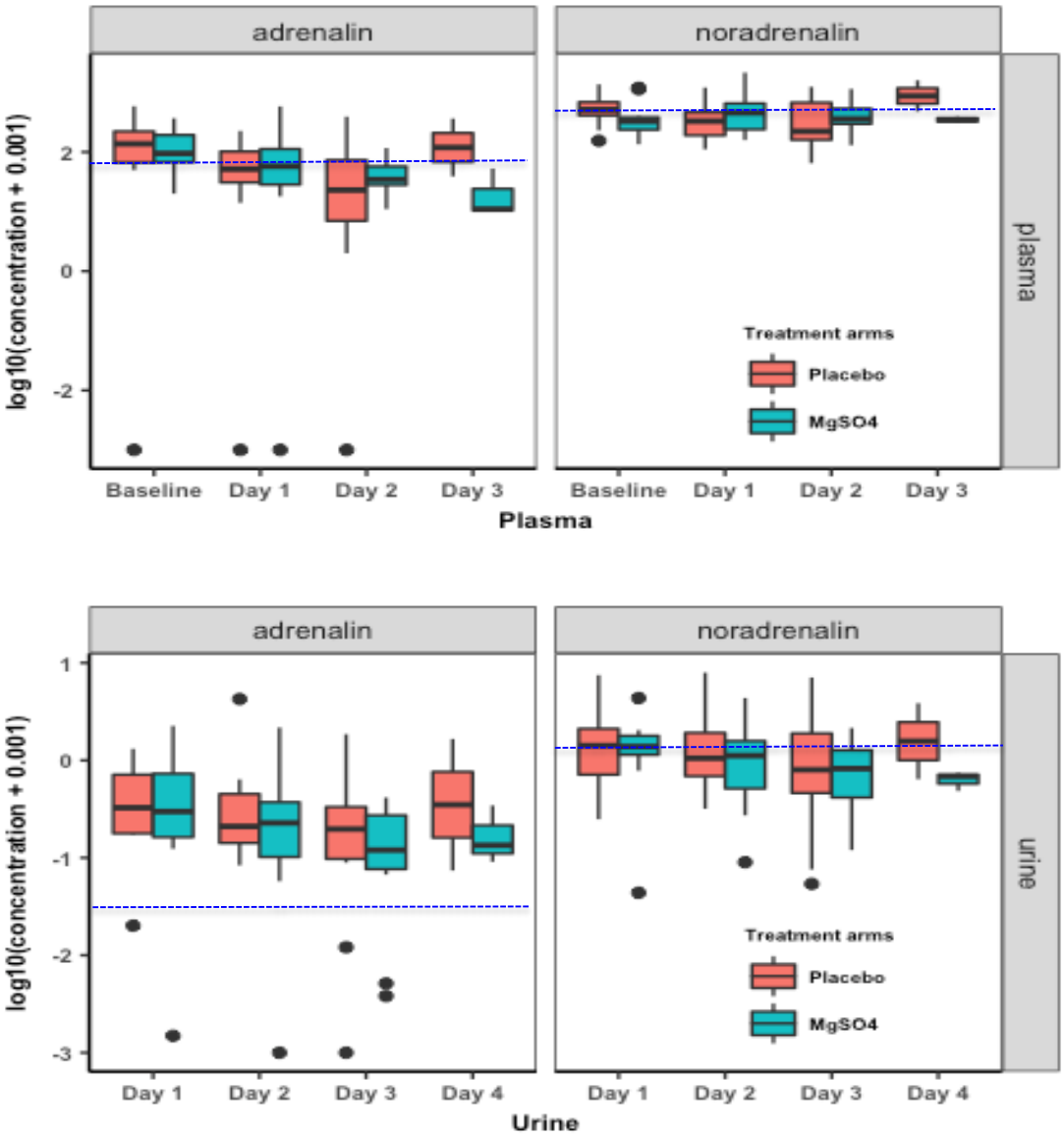
					Comparison by linear mixed effects regression		
	Placebo (N=12)		MgSO4 (N=14)		Covariates	Mean difference (95%CI)	p-value
Plasma Adrenaline (pg/ml)*							
Baseline	12	137.5 (0.0, 590.0)	10	92.5 (20.0, 370.0)	(Intercept)	1.602 (1.028, 2.176)	0.000
Day 1	10	52.0 (0.0, 227.0)	13	58.0 (0.0, 586.0)	MgSO4	0.189 (-0.524,0.902)	0.609
Day 2	11	23.0 (0.0, 394.0)	13	34.5 (11.0, 117.0)	Time	-0.006 (-0.017, 0.006)	0.284
Day 3	2	202.5 (39.0, 366.0)	3	11.0 (10.0, 53.0)			
Plasma Noradrenaline (pg/ml)*							
Baseline	12	520.5 (155.0, 1372.0)	10	334.5 (135.0, 1195.0)	(Intercept)	2.615 (2.450, 2.780)	0.000
Day 1	10	330.0 (111.0, 1213.0)	11	459.0 (159.0, 2166.0)	MgSO4	0.000 (-0.213,0.213)	0.997
Day 2	11	223.0 (65.0, 1250.0)	12	359.0 (130.0, 1152.0)	Time	-0.001 (-0.214, 0.212)	0.466
Day 3	2	1042.5 (479.0, 1606.0)	3	348.0 (305.0, 396.0)			
Urine Adrenaline (μg/kg/24hr)							
Day 1	10	0.326 (0.019, 1.315)	11	0.297 (0.000, 2.261)	(Intercept)	-0.336 (-0.743, 0.072)	0.113
Day 2	12	0.210 (0.083, 4.257)	14	0.237 (0.000, 2.173)	MgSO4	-0.130 (-0.545,0.286)	0.545
Day 3	12	0.196 (0.000, 1.857)	14	0.121 (0.003, 0.415)	Time	-0.007 (-0.012, -0.001)	**0.032**
Day 4	2	0.866 (0.073, 1.658)	3	0.133 (0.090, 0.344)			
Urine Noradrenaline (μg/kg/24hr)							
Day 1	10	1.439 (0.247, 7.503)	11	1.384 (0.043, 4.355)	(Intercept)	0.273 (-0.011, 0.557)	0.065
Day 2	12	1.059 (0.317, 7.963)	14	1.114 (0.089, 4.368)	MgSO4	-0.082 (-0.376,0.212)	0.589
Day 3	12	0.815 (0.053, 7.091)	14	0.828 (0.019, 2.152)	Time	-0.005 (-0.008, -0.001)	**0.013**
Day 4	2	2.250 (0.638, 3.863)	3	0.682 (0.483, 0.743)			

Note: Assay sensitivity for a) plasma adrenaline: 5 pg/ml, b) plasma noradrenaline: 16 pg/ml, c) urine adrenaline: 0.08 ng/ml, d) urine noradrenaline 0.24 ng/ml

Summary statistic is median (range) for all parameters. Significant p-values shown in red bold face type.

* Missing data in 8 samples due to initial test failure and insufficient plasma to repeat the test.

Figure 4.4: Boxplots showing the evolution of plasma and urine catecholamine levels over time in the two study arms.



Dotted blue lines represent the upper limit of normal in children for the respective measurements.

Since the distribution of the catecholamine levels was skewed the data were log-transformed. Since levels of some samples were below the assay sensitivity level, 0.001 was added to each result to avoid a final result of infinity when taking the log10 value of 0.

4.3.7 Correlations between catecholamine levels and hemodynamic parameters

HR, SBP and MAP values correlated to varying degrees with plasma adrenaline and noradrenaline concentrations, but none of the relationships were strong (Table 4. 10). The relationship between increasing HR with higher plasma adrenaline levels [rho (95%CI) of 0.33 (0.17, 0.48), p<0.001],] was slightly stronger than for the other parameters, SBP and MAP. Relationships between plasma noradrenaline and the three cardiovascular parameters were rather similar, all with adjusted correlation coefficients around 0.3.

Relationships between urine catecholamine levels and the different cardiovascular parameters were less convincing.

Table 4.10: Relationships between catecholamine levels and HR, SBP, and MAP adjusted for treatment arm and timing of sample

	rho	(95% CI)*	p-value
Plasma Adrenaline (pg/ml)*			
HR (beats/min)	0.33	(0.17, 0.48)	<0.001
SBP (mmHg)	0.23	(0.08, 0.38)	0.003
MAP (mmHg)	0.20	(0.01, 0.38)	0.040
Plasma Noradrenaline (pg/ml)*			
HR (beats/min)	0.32	(0.14, 0.48)	<0.001
SBP (mmHg)	0.32	(0.09, 0.52)	0.008
MAP (mmHg)	0.30	(0.08, 0.49)	0.007
Urine Adrenaline (μg/kg/24hr)			
HR (beat/min)	-0.08	(-0.39, 0.25)	0.644
SBP (mmHg)	0.13	(-0.20, 0.44)	0.445
MAP (mmHg)	0.21	(-0.23, 0.57)	0.351
Urine Noradrenaline (μg/kg/24hr)			
HR (beats/min)	0.18	(-0.11, 0.44)	0.230
SBP (mmHg)	0.20	(-0.12, 0.48)	0.216
MAP (mmHg)	0.20	(-0.14, 0.50)	0.251

rho = Pearson's correlation coefficient, adjusted for treatment arm and time of sample from study enrolment

4.4 Discussion

Therapeutic options for severe HFMD remain limited [83, 117, 168], despite the rapidly increasing burden of disease in the Southeast Asian region over the last two decades, and the life-threatening nature of the clinical syndromes seen in a small proportion of cases [97, 192, 227-229]. ANS dyregulation is one of the key features of severe disease, indicating brainstem involvement, and early recognition of the associated hypertension is thought to be essential to successful management. However, there is a lack of good evidence on how to control hypertension in severe HFMD.

During the recent major epidemic in southern Vietnam, at a time when we were seeing large numbers of severe cases, I developed the protocol for this trial investigating the efficacy of MgSO4 in severe HFMD [98]. Use of a novel treatment in severe patients who have failed on conventional treatment is accepted as reasonable by most clinicians faced with difficult decision-making in such circumstances. However conducting a randomized blinded trial in severely ill children requires a cautious approach with a strong focus on safety, in order to convince ICU staff that it is ethical to allow their patients to be enrolled. Although there was some safety data on use of therapeutic effect of MgSO4 in young children in other diseases [176, 230, 231], this would be the first use in a blinded trial in severe HFMD. In the early stages of the discussion about the trial with MoH, there was a feeling among some members of the Vietnamese Guidelines Committee that MgSO4 should be introduced directly into the guidelines on the strength of the evidence from the preliminary pilot series data. Eventually I was able to persuade MoH that a formal RCT investigating intervention with MgSO4 at a less serious stage of the illness with safety as the priority was acceptable ethically, and that it was also necessary and important to demonstrate clearly whether the observed effect was real. Then the trial was designed very carefully to ensure there would be no treatment delay for the children and that all possible safety mechanisms were incorporated in the protocol. All these activities took over a year to complete so that the study did not actually start until mid-2014, by which time the epidemic in the region was waning and the number of severe cases was declining.

This may have been partly due to changes in the HFMD epidemiology across the region, with a reduction in the number of cases with EV-A71 infection.

The trial started first at one study site, the PICU at HTD, so that I could personally ensure that all the SOPs and study procedures worked well, and that seriously ill children with the potential to deteriorate rapidly, could be rapidly enrolled without compromising their rights. We also had an independent DSMB who reviewed all the data after the first 5 cases had enrolled, and again after 20 cases had enrolled. In addition MoH conducted an audit during the early phase of the trial and reviewed all AEs and SAEs. Inevitably with a trial of this complexity not all eligible participants were enrolled. At HTD, where I was personally on site most of the time, we were able to recruit 26 patients before the disappearance of HFMD locally. However, when the trial opened at CH1, which is a much bigger and busier hospital seeing more than double the caseload of HTD, it became clear that the rapid recruitment protocols were not working. In 10 of the cases screened at this site the main reasons for failure to recruit were worsening hypertension with failure to access an arterial line, both probably related to staff workload. After making efforts to improve recruitment at this site, we considered opening a third site in the Mekong Delta but by this time it was apparent that the sample size of 190 patients could not be achieved before the disease disappeared.

For the 26 cases enrolled at HTD compliance with all aspects of the study protocols was very good, with only a small number of minor protocol deviations and very good data quality overall. Also all 26 children attended the follow-up visit after 6 months, although two did not cooperate with the neurodevelopmental testing. After the decision to stop the trial was taken, together with the OUCRU statisticians I wrote a formal analysis plan taking into account the very small number enrolled compared to the initial sample size calculation (which itself was based on a large postulated effect size). The primary analysis was by intention-to treat according to the randomized arm. We decided however, that because of the very low rate of non-compliance a per-protocol analysis was not necessary, and that the various planned analyses by

individual outcome and/or by enterovirus serotype should be dropped due to the small numbers.

Baseline information was quite similar between two treatment arms, with only minor differences apparent in most of the characteristics assessed. However there was a clear difference in the proportion enrolled with Stage 2 hypertension, a feature associated with more severe disease, with 8/12 (67%) enrolled at this stage in the placebo group versus 3/14 (21%) in the MgSO4 group. Median systolic and diastolic pressures were also marginally higher in the placebo group but these values were not age-adjusted. Conversely, EV-A71, the serotype that has generally been associated with more severe outcomes [112, 208], predominated in the MgSO4 group. However, the number of cases with negative enterovirus PCR was greater (5/12, 42%) in the placebo group compared to 3/14 (21%) in the MgSO4 group; although these may be true negatives, a number of factors including sample quality, transport, and processing may have affected the sensitivity of the PCR. Overall, these differences between the treatment arms are likely to be random and related to the small number of participants recruited [232], but of course there is the potential for an effect on the outcomes of interest.

Although the initial sample size was based on efficacy measures, the trial was set up with as strong emphasis on safety. Because of the small number of patients enrolled and concerns about random error in small trials [232], we did not do formal statistical comparison of the AE and SAE rates between the treatment groups. However, the numbers and types of both clinical and laboratory events were similar in the two groups. One particular concern about potential side effects of MgSO4 is development of respiratory muscle weakness when plasma levels exceed 3 mmol/l [233]. In this trial, to be cautious I chose an upper limit of 2.5 mmol/l as the safety level at which the independent should recommend titrating the infusion rate downward. However a higher level of 4 mmol/l was used in the tetanus trial performed here some years ago [171], but the patients were all adults who had tracheostomies, meaning that the doctors would be able to respond rapidly to deteriorating respiratory status. Levels

from 3.5 to 5.5 mmol/l were targeted in a trial assessing the efficacy and safety of MgSO4 in severe persistent pulmonary hypertension in neonates [176], but all these cases were already ventilated.

Severe HFMD can cause significant neurological sequelae in survivors, but it is also important to investigate possible negative effects on the long-term development of children treated with MgSO4 during the severe acute phase. A small number of study participants did have clinically detectable neurological abnormalities at discharge that persisted for a few months, but all appeared to have recovered fully by 6 months. To be more detailed we performed neurodevelopmental assessments after 6 months using the Vietnam adapted Bayley-III scale. In the initial testing carried out at or soon after discharge there were minor differences in the expressive language domain of the tool, but there were no differences between the MgSO4 and placebo groups at 6 months. Sedation may have affected the ability of some children to perform the tests around discharge, but a random effect due to the small number of study participants and multiple statistical analyses is also possible.

No long-term detrimental neurodevelopmental effects were reported in the small study of neonates treated with MgSO4 for persistent pulmonary hypertension previously mentioned [176], with normal developmental assessments documented in these 11 infants after 6 and 12 months. However, a neuroprotective effect in neonates was noted, in a large meta-analysis assessing the safety and efficacy of MgSO4 given to women at high risk of pre-term labour; a reduction in the rate of moderate to severe cerebral palsy was found in the babies of mothers who received MgSO4 compared to those who did not, in this meta-analysis involving almost 20,000 preterm infants, although mortality was similar between the groups [231], In contrast, no improvement in clinical outcome after one month, based on death or a modified Rankin Score of 4-5, was found among 1204 adults enrolled in a randomized placebo-controlled trial assessing use of MgSO4 for aneurysmal sub-arachnoid haemorrhage [234]. In our trial the numbers were too small to assess the efficacy of MgSO4 on neurodevelopmental outcomes after severe HFMD, but from the safety perspective the results are

reassuring. To evaluate neuroprotective efficacy, a much larger study population would be needed, as was originally intended.

Altogether, reports of AEs in children associated with use of MgSO4 have not been common up to now, for example in the Cochrane meta-analysis of the effect of MgSO4 in acute asthma in children, the long-term follow up of children in the Magpie trial (a randomized placebo-controlled trial of MgSO4 in around 7,000 women with pre-eclampsia), or the neuroprotection meta-analysis for preterm neonates described above [221, 231, 235]. However many of the authors concluded that the data on AEs was not sufficient to make firm conclusions about whether MgSO4 is safe or not, especially in children with asthma. Of note, increased toxicity was observed in the mothers in the meta-analysis of MgSO4 use in women at high risk of preterm labor, but with considerable heterogeneity between individual studies; respiratory depression did occur in the mothers although there were no significant differences in terms of AEs in their infants [231]. Therefore the potential for side effects with MgSO4 remains a concern both in clinical practice, and for future clinical research studies.

With respect to efficacy, although we observed minor differences in the findings for a number of parameters generally favouring the MgSO4 group, there were no significant differences in any of the primary and secondary outcomes evaluated. However, given the small number of patients enrolled and the low event rate, no real conclusions can be drawn. The initial pilot data had suggested an effect in patients with Stage 2 hypertension who were already on milrinone, and it is possible that among the less severe cases enrolled in the RCT, that any potential beneficial effect was diluted. Baseline severity was generally similar in the treatment arms although a smaller proportion of the MgSO4 group had Stage 2 hypertension at enrollment. Also it may be that the dose we used in this RCT was not effective. We chose a cautious approach given that we anticipated enrollment of large numbers of children in a short space of time, who were breathing spontaneously. Higher dosage might put them at risk of respiratory depression, potentially requiring intubation and ventilation, which might increase the risk of a bad outcome for the individual child and would seriously stretch

the resources of our PICU. Aiming for a higher plasma Mg level as was done in the tetanus trial might have proved more effective in controlling hypertension [171]. Finally the domination of EV-A71 as the etiological agent causing infection had changed by the time we were able to commence recruitment, and there is general recognition that other enterovirus serotypes are less likely to cause very severe disease.

Catecholamine levels (both adrenaline and noradrenaline) in urine decreased significantly during the course of the study, but without a clear change in corresponding plasma catecholamine levels. No differences were seen in either plasma or urine measurements between the treatment arms, but given the small numbers in each group this was to be expected. Studies in pregnant and non-pregnant rabbits have shown that MgSO4 reduces plasma adrenaline levels in parallel with reducing MAP [236]. In adults with tetanus, those treated with MgSO4 had significantly less ANS dysregulation, required less cardiovascular stabilizing drugs and had lower urinary excretion of adrenaline but higher excretion of noradrenaline [172]. In a case series of 17 individuals with phaechromocytoma, use of MgSO4 during induction of anaesthesia resulted in satisfactory control of cardiovascular parameters in 15 cases, although four patients also received nitroprusside. Catecholamine release was studied in 5 of the patients and indicated that MgSO4 use was associated with reduced catecholamine concentrations [237]. Lastly, catecholamine storm is thought to be the mechanism underlying the Irukandji syndrome (caused by envenomation by Carukia barnesi, a kind of jellyfish) for which MgSO4 is often used as rescue treatment in Australia. However in a systematic review carried out in 2017 the authors found no clear evidence to support treatment with MgSO4 and concluded that additional research is needed to confirm or refute the current practice [238]. An association between catecholamine levels, particularly adrenaline levels, and cardiovascular parameters is thus supported by several lines of evidence, but considerable debate continues around the question of whether intervention with MgSO4 influences either hypertension related to ANS dysregulation or the associated catecholamine pathway derangements.

4.5 Conclusion

This was the first randomized controlled trial of a novel treatment for hypertension secondary to ANS dysregulation ever carried out in young children with severe HFMD. It was started in the later stages of a major HFMD epidemic across the region, at a time when there was heightened public awareness and anxiety about this disease, resulting in huge pressure on healthcare services.

The trial was designed and executed very carefully, with a major emphasis on safety for the participants, and it is likely that this did slow down recruitment. Obviously, the low rate of recruitment, with only 26 patient enrolled before the disease disappeared, adversely affected the power to ascertain efficacy, but we learned a great deal about how to develop and manage such a trial in difficult circumstances and generated valuable safety data. It seems likely that further epidemics of HFMD will continue to occur at intervals across the Asian region, and that at some point in the future clinicians will again face the difficulty of managing young children with severe disease with brainstem involvement and ANS dysregulation.

Although we found no evidence of benefit in this trial involving a very small number of children, it is clear that these data are not sufficient to address the important question originally posed, and that a much larger trial is still needed to properly evaluate whether MgSO4 has a role in controlling the BP in severe HFMD. The experience gained from this trial, both the general experience of conducting a complex trial in difficult circumstances, and the more particular information regarding the positive safety profile of MgSO4 used in this way plus the information generated on dosing in relation to plasma Mg/Ca levels, should prove invaluable if and when the next epidemic occurs. A well-designed protocol is already available, together with detailed SOPs to guide implementation, hopefully allowing all the necessary steps to set up a new RCT to take place rapidly, ideally within the first 6 months of the emergence of another epidemic in the region.

Chapter 5

MgSO4 IN SEVERE HFMD: THE RETROSPECTIVE COHORT ANALYSIS

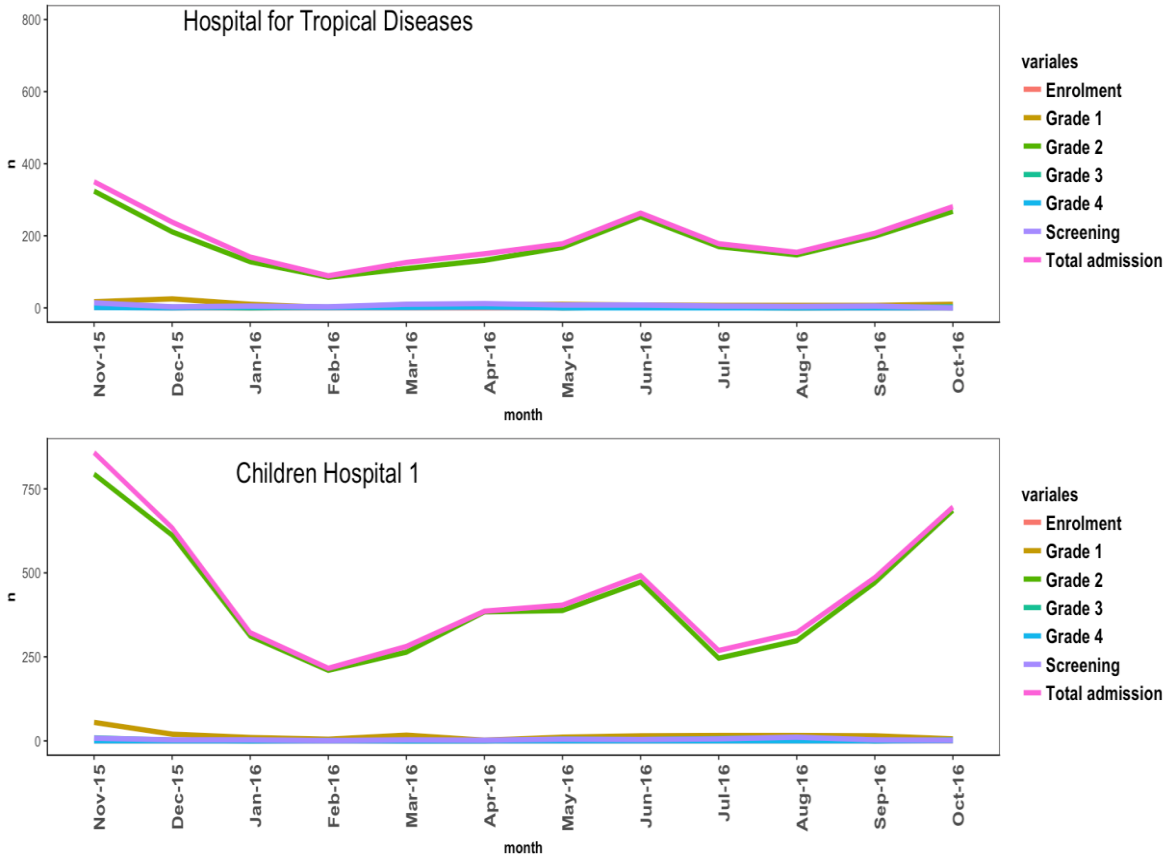
5.1 Introduction

As described previously, the phosphodiesterase-3 inhibitor, milrinone, is currently recommended in the Vietnamese MoH guidelines for management of HFMD patients with brainstem encephalitis who develop ANS dysregulation and Stage 2 hypertension. However, in practice a number of children continue to deteriorate despite maximum dose milrinone. Through close observation of severe HFMD cases and extrapolation from my experience with neonatal tetanus cases I postulated that MgSO4 might be beneficial in these severe cases, and therefore began giving this treatment empirically to those for whom milrinone failed to control blood pressure from the end of 2011/early2012. Initial results of using MgSO4 as second line therapy in this way appeared promising and this approach was increasingly adopted for severe cases during 2012. During 2013 I developed the protocol for the randomized controlled trial described in Chapter 4 and recruitment commenced in June 2014. Unfortunately however by then the numbers of HFMD cases being seen in Ho Chi Minh City and generally across the region had started to decrease, and by the end of 2015 severe case numbers were very small (Figure 5.1). By September 2016 there were so few cases being enrolled that the trial was stopped. As can be seen almost all cases admitted from November 2015 onwards at the two hospital sites had Grade 2 disease and did not progress to Grade 3 disease.

In the RCT the criteria for enrolment and randomization to receive either MgSO4 or placebo in a double-blind manner were deliberately set at the lower level of hypertension (Stage 1) so as to allow for the existing MoH guidelines to be applied if the SBP reached the Stage 2 hypertension level. However, during the stage of protocol development and study set up I continued to use MgSo4 as second line therapy for

severe cases with Stage 2 hypertension who were already on high dose milrinone. In addition, after commencing the RCT there were several severe cases

Figure 5.1: Total HFMD admissions, and screening/enrolment numbers for the MgSO4 RCT, at the two HCMC study sites from Nov 2015 to Oct 2016, with a breakdown by discharge severity Grade.



that did not fit the study enrolment criteria or where the family declined to participate, for whom MgSO4 was also given a second line therapy. Apart from the basic difference in clinical severity between the trial population (Stage 1 hypertension without milrinone) and the open-label MgSO4 recipients (Stage 2 hypertension already on high dose milrinone) the same general approach to treatment was in operation by the same core group of PICU clinicians throughout this period.

Since the trial was stopped early and the results were inconclusive I decided to review the information available for all HFMD cases managed on PICU between 2011 and 2015 who were treated with MgSO4 outside the trial. I also identified a group of patients of similar severity who had not been treated with MgSO4 during this period, in order to compare a variety of endpoints with the MgSO4 group. In this chapter I will present the methodology and findings from this retrospective analysis.

5.2 Materials and methods

5.2.1 Study design

This was a retrospective observational cohort study to assess the response to open-label intravenous MgSO4 used in Vietnamese children with Grade 3 HFMD and signs of ANS dysregulation with Stage 2 hypertension. I wished to compare responses in patients who fulfilled these criteria and received MgSO4 (the exposed group), with similar cases who achieved the same basic severity level but for whom MgSO4 was not used (the control group), particularly during the transition period when this treatment was considered novel.

5.2.2 MgSO4 regimen

The regimen used in the exposed patient group was basically similar to that used in the trial – with a loading dose of 50mg/kg $MgSO_4$ diluted to 10% in sterile water and given by continuous infusion into a peripheral intravenous line over 20 minutes, followed by a maintenance infusion of 30-50 mg/kg/hr according to response. This dosing schedule was selected because the clinicians on PICU were familiar with it from their management of neonatal tetanus cases. It is also similar to the regimen recommended at the Royal Children's Hospital in Melbourne for treatment of severe exacerbations of asthma [239].

As this was a novel treatment being given to very sick children, there was a strong emphasis on safety monitoring. Plasma Mg/Ca levels were measured routinely after 6 and 12 hours and then daily if stable, with the clinicians aiming to raise plasma Mg

levels to around twice normal (1.8mmol/l). By the time the RCT described in Chapter 4 started the staff were comfortable that clinical signs of toxicity were unlikely at this level, and they were therefore happy to increase the infusion rate to the higher range of 50mg/kg in most cases, aiming not to exceed the upper limit of 2.5 mmol/l for plasma Mg rather than to achieve the lower limit of 1.8 mmol/l.

5.2.3 Standard clinical and laboratory protocols in operation 2011-2015

The Vietnamese MoH guidelines specify patient care protocols for a variety of conditions. Based on the WHO 2010 HFMD guidelines and local VN guidelines from mid-2011, HFMD patients graded as 2b or above should be managed in an environment with the capacity to carry out detailed clinical observations every 1 to 3 hours, increasing to every 30-60 minutes in the event of deterioration. Detailed guidelines also exist for most treatment strategies, including specific indications for drug therapy (IVIG, milrinone etc.) as well as practical interventions such as ventilation and hemofiltration (see Appendices, page 210).

In addition the guidelines recommend a schedule for laboratory investigations in severe HFMD patients. Blood glucose, FBC and CRP should be measured routinely in all HFMD cases admitted to HDU/PICU, with other tests left to the discretion of the treating clinician. Collection and storage of diagnostic swabs for enterovirus PCR became mandatory for all HDU/ICU cases during 2011.

5.2.4 Identification of study subjects

The HTD main database records basic information on all patients admitted to the hospital, including information on all medications prescribed. From this database I extracted the hospital file numbers of all patients who had received milrinone over a 5-year period from January 2011 to December 2015. I then requested these files and checked each record to confirm that the patients had been admitted to PICU with a clinical diagnosis of HFMD, and that they had not been enrolled in the MgSO4 RCT.

I then reviewed the selected hospital files and determined which patients did not respond to milrinone according to the following criteria, and also the date/time when they reached this severity level.

- SBP persisting above Stage 2 hypertension (VN MoH guidelines) for at least 60 minutes after commencing maximum dose milrinone, i.e. 0.7 µg/kg/minute
- SBP increasing rapidly (by more than 10 mm Hg above the MoH threshold) after reaching a dose of at least 0.6 µg/kg/minute milrinone, or within 30 minutes of moving up to 0.7 µg/kg/minute

I also checked that the patients a) had not received fluid resuscitation or inotropes such as dopamine, adrenaline or noradrenaline during this time-period or previously during the hospital admission, and b) had not needed any other supportive therapy such as ventilation or haemofiltration during this time-period or previously.

I selected all cases who fulfilled these criteria for detailed file review and data extraction. For the MgSO4 group the date/time of starting the drug was taken as T=0, while for the control subjects T=0 was the time when they would have been eligible to start MgSO4 according to the above criteria.

5.2.5 Data collection and data management

I developed a specific case report form (CRF) for this study focused on documenting all available information on cardiovascular parameters in these patients. The CRF included baseline information from T=0 and then hourly nursing observations for a minimum of 24 hours from that time, then with reduced frequency for up to 72 hours or until recordings in the hospital files stopped. In addition data for up to 6 hours prior to T-0 were extracted from the files, provided the patient was in PICU during this period.

After training using the study specific CRF, research nurses who were familiar with extracting data from hospital files collected the data. I crosschecked and corrected the paper CRFs based on the hospital files, together with several PICU colleagues, and the

study nurses then entered the information into an electronic database. After completing single data entry, the electronic data were checked to identify outliers or implausible values and traced back against the clinical files and/or the hospital's electronic clinical and laboratory databases.

These children were also included in the main clinical observational study described in Chapter 3. That database included once daily assessments during the PICU stay, with information on clinical signs, particularly neurological features and cardiovascular and respiratory manifestations, collected from the routine daily ward round documentation. Information on ANS manifestations such as mottled skin, sweating etc. was not systematically recorded in the hospital files during this period, but any information noted in the files was collected.

5.2.6 Statistical analysis

Patients who received MgSO4 for hypertension were considered as the exposed patients, while those who did not receive MgSO4 but may have received alternative second line therapy, were considered as unexposed and formed the control group.

Major events: My intention was to examine a composite endpoint comprising progression to any major clinical event within the first 72 hours after T=0; the endpoints were shock, need for inotropic support, respiratory compromise requiring ventilation, or death. Comparison between the groups was based on logistic regression with the non randomized treatment assignment as the only covariate. However, there were no deaths and very few major events in either patient group. I also assessed the duration of milrinone therapy and of hospitalization after T=0, using linear regression.

Ten deaths did occur within the specified time period, 9 of them during 2011 and one in January 2012 (Table 3.4, Chapter 3). Among these patients, 6 had hypertension but deterioration was generally rapid, progressing to pulmonary edema and/or shock before they reached the maximum dose of milrinone. Thus none of the 10 patients fulfilled the selection criteria for inclusion in this analysis.

Comparison of hemodynamic parameters between treatment groups: I also wanted to compare the magnitude and time course of hemodynamic stabilization over the 24 hours after T=0 in the two groups to see if blood pressure was more effectively controlled in the MgSO4 group. For this analysis I focused on the SBP and the mean arterial pressure (MAP) calculated in a standard way as follows: [SBP+2(DBP)]/3.

Examination of the MgSO4 group data showed that the actual time of starting MgSO4 in this group was quite variable in relation to the apparent T=0 as defined in the selection criteria described earlier. A number of factors may have influenced this clinical decision-making including the age of the patient, the rapidity of the deterioration in blood pressure, and the experience of the clinician involved. For this detailed analysis of hemodynamic parameters and so as to allow for the intrinsic variability in the initiation time in the MgS04 group, I decided to develop a prediction model for MgSO4 initiation after consultation with experienced statisticians at OUCRU. This model was then used subsequently as an imputation model to create multiple imputed datasets that defined multiple presumptive time points for when MgSO4 might have been initiated in the control group.

This prediction/imputation model is a logistic regression model based on three factors: a) the difference between the current SBP and the age-dependent cut-off for Stage 2 hypertension; b) the difference between the current SBP and the previous SBP value; and c) the current dose of milrinone. For control patients, at each time-point when the SBP was measured and was larger than the age-dependent cut-off, a probability of initiating MgSO4 was estimated from the imputation model. For each patient, the imputed time of MgSO4 initiation was the first instance when MgSO4 would be used, based on random assignment using a Bernoulli distribution where the probability of success is the probability of initiating MgSO4 estimated from the imputation model. A patient was considered as not receiving MgSO4 if it was not initiated at all time-points based on these probability models. This procedure was repeated 20 times for each patient to create 20 imputed datasets.

Although MgSO4 was initiated at Stage 2 hypertension in the exposed group after failure with milrinone, this is a rather high BP level and Stage 1 hypertension is more relevant as a clinical threshold for concern and potential intervention. Therefore I examined the area under the curve (AUC) for all SBP and the MAP values above the appropriate age-dependent Stage 1 hypertension threshold during the first 24 hours in the two groups, comparing values after actual T=0 in the exposed MgSO4 group and presumptive time points for initiating MgSO4 in 20 imputation datasets in the control group.

Relationships between Mg/Ca levels and hemodynamic parameters: Associations between sequential Mg/Ca levels and SBP and MAP were explored using scatterplots and Pearson correlation coefficients.

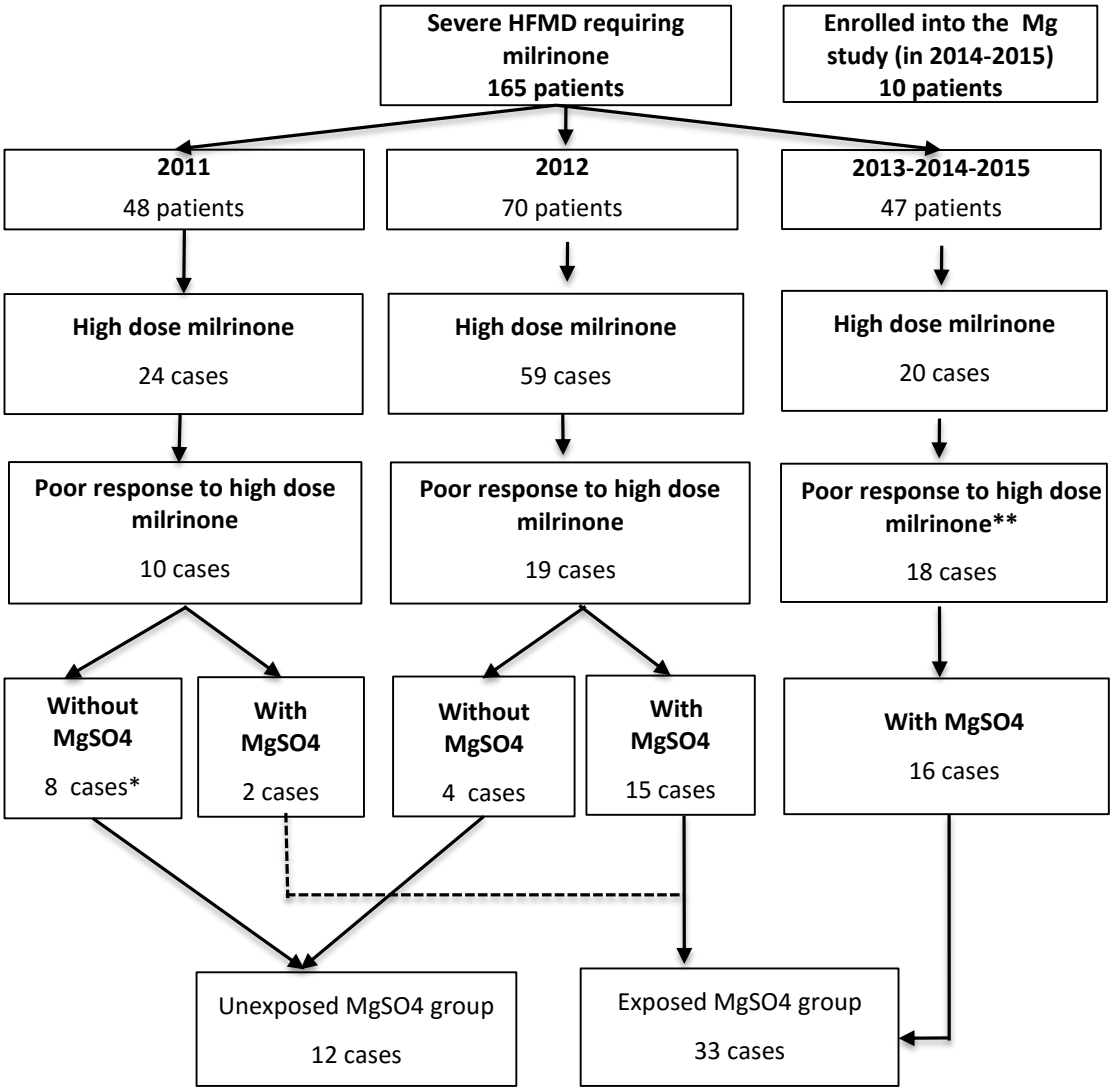
5.3 Results

From January 2011 to December 2015, 165 children with severe HFMD were admitted to the PICU at HTD and treated with milrinone, and therefore were potentially eligible for inclusion in this analysis (Figure 5.2). In this group, 103/165 (63%) required high dose milrinone for control of hypertension, and in 47/103 cases the blood pressure remained high or continued to increase despite being on the maximum dose of the drug. Within this group however, one case had already commenced nicardipine before the addition of milrinone, and in another case MgSO4 was added due to severe asthma rather than for hypertension; these cases were not included in the analysis. A total of 45 cases were selected, 33 in the group exposed to MgSO4 and 12 in the unexposed (control) group. One child in the control group developed respiratory distress and was ventilated, but subsequently the blood pressure remained poorly controlled and magnesium was added at this time. All blood pressure measurements after addition of MgSO4 were censored from this point onwards.

In all 33 children in the exposed group the MgSO4 infusion was continued for at least 24 hours. By 48 hours the infusion had been stopped in 8 cases, and by 72 hours it had been stopped in 18 cases. Among the patients in the control group alternative therapy for hypertension was added only in the one case described above, who was ventilated

7 hours after reaching maximum dose milrinone, and then 1 hour later MgSO4 was added for poorly controlled hypertension.

Figure 5.2: Flow chart describing selection of patients for inclusion in the analysis



*: 1 case was eventually treated with MgSO4 but only some time later, after being ventilated

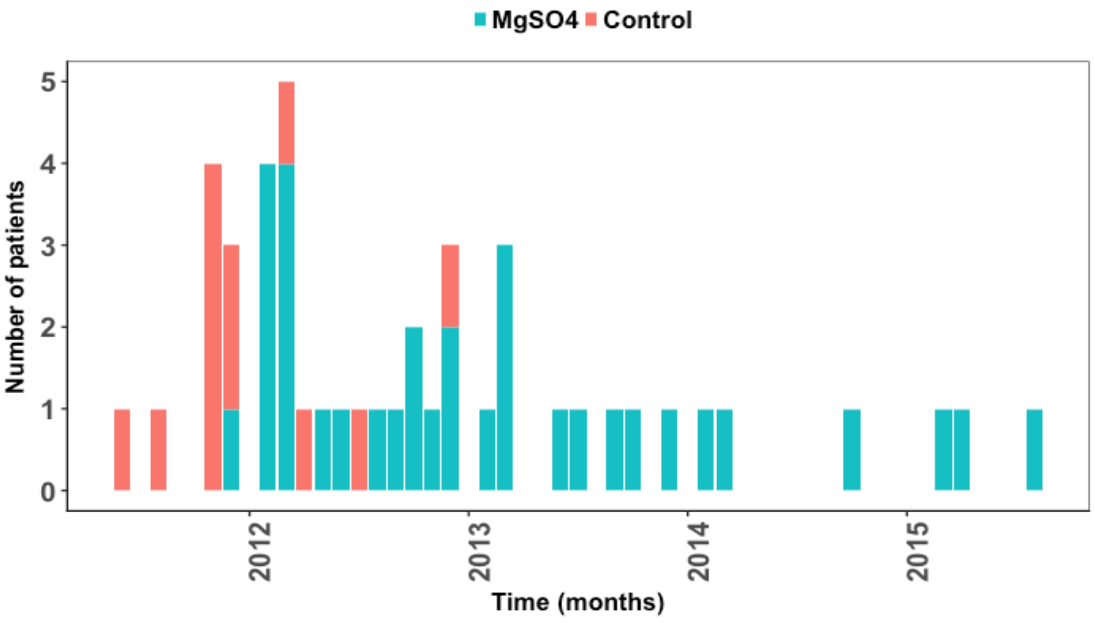
**: 1 case had received nicardipine before adding MgSO4 and 1 case received MgSO4 because of severe asthma manifestation

5.3.1 Baseline clinical and laboratory information for the two groups

Most patients (8/12, 67%) in the control group were hospitalized during 2011, the first year of the major outbreak, with hospital admission for the remaining 4 children distributed intermittently through 2012. MgSO4 was first given at the end of 2011 and by mid-2012 had become an accepted therapeutic intervention. From then onwards almost all HFMD cases who did not respond to milrinone were treated with MgSO4 (Figure 5.3).

Baseline demographic, clinical and laboratory information comparing the subjects in the exposed and control groups are shown in Table 5.1. For most continuous parameters the data presented are the worst values documented within the 24 hour period prior to T=0, except for the illness day and the blood pressure values, which correspond to T=0.

Figure 5.3: Temporal distribution of hospital admissions between 2011 and 2015, for patients in the two groups



Note: MgSO4 exposed patients are shown in red, and control patients in blue.

Table 5.1: Clinical and laboratory features in the MgSO4 exposed and control groups, assessed within the 24 hours before the actual/potential time to start MgSO4 (T=0)

	n	MgSO4 (N=33)	n	Control (N=12)	Estimated Effect (95% CI) #	p-value &
Demographic information						
Sex (F)	33	7 (21)	12	6 (50)	0.27 (0.06, 1.10))	0.067
Age (months)	33	36 (25, 48)	12	15 (12, 21)	22.43 (6.67, 38.20)	0.005
Weight (kg)	33	18 (14, 24)	12	10 (9, 11)	9.97 (4.69, 15.25)	<0.001
Illness day at T=0	33	4 (3, 4)	12	4 (3, 6)	-1.04 (-2.17, 0.09)	0.062
Admission grade*	32		12		#	0.797
- 2		0 (0)		3 (25)		
- 2b		19 (59)		9 (75)		
- 3		13 (41)		0 (0)		
Clinical manifestations						
Fever (> 37.5^0 C)	33	26 (79)	12	11 (92)	0.34 (0.02, 2.23)	0.288
Tachycardia**	33	7 (21)	12	2 (17)	1.35 (0.27, 10.07)	0.732
Systolic BP at T=0	33	140 (130, 147)	12	134 (128, 137)	7.04 (-3.43, 17.50))	0.170
Diastolic BP at T=0	33	70 (63, 80)	12	64 (60, 69)	6.51 (-1.77, 14.79)	0.110
Tachypnea for age	29	16 (55)	12	9 (75)	0.41 (0.08, 1.71)	0.227
Irregular breathing	33	3 (12)	12	2 (18)	0.61 (0.09, 5.25)	0.628
Skin ANS features***	33	1 (3)	12	0 (0)	#	0.428
Laboratory investigations						
WBC ($x10^9$/L)	30	12.6 (10.8, 16.1)	10	12.0 (8.8, 14.2)	-2.73 (-5.49, 0.03)	0.045
Neutrophil (%)	30	58.8 (48.4, 69.4)	10	47.5 (43.8, 58.4)	10.30 (0.06, 20.54)	0.042
Hb (g/l)	30	11.9 (10.9, 13.1)	10	13.1 (11.9, 13.7)	-0.66 (-1.85, 0.53)	0.252
CK-MB (UI/l)	15	23.9 (20.8, 35.5)	6	36.8 (20.1, 72.6)	-28.59 (-57.19, 0.01)	0.038
Troponin I (pg/ml)	15	11.0 (5.5, 18.0)	6	13.0 (1.2, 65.2)	-133.1 (-26.97, 293.23)	0.078
Glycemia (mg/dl)	25	106 (96, 127)	9	103 (82, 111)	4.67 (-16.91, 26.26)	0.644
EV-A71 positive	33	23(70)	12	12(100)	#	0.007

Summary statistic is absolute count (%) for categorical variables and median (IQR) for continuous data

Estimated effect: OR for categorical variables (based on logistic regression model) and mean difference (based on linear regression model)

*: One case was diagnosed with encephalitis at admission

**: Heart rate sustained >150 beats/min, adjusted down by 10 for each 1oC of fever above 37.0 ^{o}C

***: Skin manifestations of autonomic nervous system dysregulation

#: cannot be compared because there was no event in one group

The proportion of girls (6/12, 50%) was higher in the control group than in the exposed group (7/33, 21%), and the children in the MgSO4 group were significantly older, with a median (IQR) age of 36 (25, 48) months versus 15 (12, 21) months in the control group, estimated effect size 22.43 (6.67, 38.20), p=0.005. Reflecting this fact, the weights of the MgSO4 subjects were also significantly greater than the weights of the control group (p<0.001). The illness day at T=0 was similar for the two groups. However, regarding clinical severity at hospital admission the control patients were generally less severe at this time (no cases were admitted at Grade 3 compared to 13/33 (41%) of the MgSO4 group), although there was no significant difference overall between the groups when assessed using ordered logistic regression.

A greater degree of family anxiety for the younger children may have resulted in earlier presentation to hospital with progression to Grade 3 occurring after hospitalization in the control group. With respect to clinical manifestations, there were no apparent differences in terms of fever, tachycardia, tachypnea, irregular breathing, and skin manifestations of ANS dysregulation between the groups. Systolic and diastolic blood were elevated in all patients, consistent with the selection criteria of Grade 3 HFMD with Stage 2 hypertension; although the values appear generally higher in the MgSO4 group these values are not age adjusted and the control patients were much younger than the MgS04 patients.

There were minor differences of borderline statistical significance in several laboratory parameters, in general indicating slightly more abnormal values in the control group compared to the MgSO4 group but the differences were small and unlikely to relevant clinically.

Diagnostic swabs were available for all 45 individuals. In 8 cases the enterovirus PCR was negative, while in the remaining 37/45 (82%) cases enterovirus infection was confirmed by RT-PCR. In the majority of these cases (35/37, 95%), the serotype was EV-A71, while in the other 2 cases no specific serotype was identified. All 12 control cases had EV-A71 infection compared to 70% of the MgSO4 subjects, probably reflecting changes in serotype dominance during the years of the study as discussed in Chapter 3.

5.3.2 Outcomes: comparison of major events between the groups (Table 5.2)

No deaths occurred in either group. Two children in the control group required invasive ventilation (for 111 and 153 hours respectively) compared to one in the MgSO4 group (100 hours) (Table 5.2). The duration of treatment with milrinone and the duration of hospitalization, both assessed in relation to T=0, were very similar between the groups.

Table 5.2: Comparison of major events occurring in the exposed and control groups

	n	MgSO4 (N=33)	n	Controls (N=12)	Estimated effect (95 % CI)	p-value [#]
Composite endpoint	33	1 (3.0)	12	2 (16.7)	0.16 (0.01, 1.79)	0.132
Invasive ventilation	33	1 (3.0)	12	2 (16.7)	0.16 (0.01, 1.79)	0.132
Duration of milrinone treatment (hrs)	33	45.5 (32.5, 67.5)	12	44.7 (34.8, 58.2)	2.42 (-17.50, 22.35)	0.802
Duration of hospitalization (days)	33	9.0 (7.0, 10.0)	12	9.0 (7.8, 13.2)	-1.65 (-3.79, 0.49)	0.117

Summary statistic is absolute count (%) for categorical variables and median (IQR) for continuous data

Composite endpoint included death, invasive ventilation, shock, and inotrope requirement.

Estimated effect: OR for categorical variables (based on logistic regression model) and mean difference (based on linear regression model)

5.3.3 Comparisons of SPB and MAP between the MgSO4 and control groups within the first 24 hours after T=0.

Development of the multiple imputation model

One patient (aged 13 years) who was much older than all the others in the MgSO4 group was not included in the development of the imputation model. All SBP data from the other 32 MgSO4 recipients excluding a) SBP values before initiation of milrinone b) SBP values after initiation of MgSO4, and c) SBP values below the age-dependent cut-off for Grade 2 hypertension, were used to develop the model. Estimated parameters for the imputation model are shown in the Table 5.3; the likelihood that MgSO4 will be given increases with a greater difference between the measured SBP and the Stage 2 age-adjusted cut-off, and with the milrinone dose >= 0.7 µg/kg/minute

Table 5.3: Estimated parameters of the imputation model

Covariate	OR	(95% CI)	p value
(Systolic BP - cut-off)	1.11	(1.05, 1.18)	<0.001
% (Systolic BP – previous Systolic BP)	1.06	(0.99, 1.13)	0.098
Milrinone dose (µg/kg/minute)			<0.001
<0.6	1.00		
(0.6, 0.7)	4.24	(0.62, 29.02)	
>= 0.7	41.89	(9.01, 194.72)	

. This model was used to create 20 imputation datasets for the control group patients (Table 5.4).

Table 5.4: Imputed time points, within the first 24 hours of achieving initial T=0, to start MgSO4 in the control patients (20 imputation datasets)

	Imputation datasets																			
ID	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**
MG012	7.00	7.00	5.00	7.00	5.00	7.00	7.00	7.00	7.00	7.00	7.00	5.00	7.00	7.00	5.00	7.00	7.00	7.00	7.00	7.00
MG037	12.25	12.25	11.42	9.25	11.42	-	12.25	12.25	11.42	-	11.42	12.25	-	6.25	18.25	12.25	-	11.42	6.25	11.42
MG038	2.75	5.75	1.00	-	-	1.00	1.00	6.75	2.75	1.00	1.00	1.00	-	1.00	2.75	1.00	5.75	-	1.00	1.00
MG042	-	-	4.50	4.00	-	4.50	-	4.50	2.00	4.50	4.50	4.50	7.00	4.50	2.00	4.00	11.00	-	4.00	4.50
MG044	5.50	3.50	1.50	4.50	21.50	3.50	1.50	2.50	2.50	2.50	2.50	1.50	12.50	5.50	2.50	5.50	5.50	1.50	12.50	12.50
MG046	22.50	18.50	21.50	22.50	18.50	22.50	18.50	19.50	22.50	19.50	22.50	21.50	18.50	18.50	19.50	18.50	18.50	18.50	21.50	18.50
MG048	-	-	35.50	35.50	-	-	30.50	35.50	26.50	45.50	35.50	11.50	35.50	35.50	35.50	14.50	35.50	45.50	26.50	20.50
MG050	8.50	11.50	11.50	8.50	7.50	7.50	7.50	7.50	8.50	7.50	7.50	8.50	11.50	7.50	7.50	7.50	7.50	7.50	7.50	12.50
MG051	2.50	4.50	0.50	2.50	2.50	3.50	0.50	0.50	2.50	2.50	2.50	9.50	2.50	5.50	3.50	2.50	3.50	2.50	2.50	0.50
MG054	15.33	12.33	9.33	12.33	12.33	13.33	12.33	16.33	12.33	18.33	13.33	10.33	12.33	15.33	13.33	10.33	12.33	15.33	12.33	15.33
MG055	19.00	40.00	17.00	15.00	15.00	32.00	32.00	19.00	19.00	-	17.00	19.00	17.00	19.00	17.00	43.00	-	40.00	19.00	32.00
MG056	2.67	2.67	2.67	3.67	2.67	2.67	2.67	2.67	6.67	2.67	2.67	2.67	2.67	6.67	2.67	12.67	14.67	2.67	4.67	2.67

Comparisons of AUCs for SBP and MAP between the two groups

During the first 24 hours after T=0, SBP and MAP measurements were missing for 60/300 (20%) potential time-points in the placebo group and for 34/825 (4%) in the MgSO4 group, most likely because the treating clinicians stressed the need for very careful observation in those receiving the new treatment. However, those missing values were imputed when calculating the AUCs. Individual AUCs for the SBP values above the Stage 1 hypertension cut-off were calculated and the difference between the MgSO4 group and each of 20 imputation datasets for the control group are shown in the Appendix. After pooling all 20 imputed datasets and deriving overall estimates using Rubin's rule, the overall difference between the AUCs, after adjustment for the "baseline" SBP recorded at the time-point of MgSO4 indication, was -46.70 (-167.77, 74.37), p= 0.450 (Table 5.5).

Similar analysis was done for the AUCs for the MAP values in the two groups. In this case there was a significant difference between the overall AUCs after pooling the results of the comparisons with the 20 imputation datasets, estimate -60.752 (-113.670, -7.835), p=0.024.

Table 5.5: Comparisons of the AUCs for SBP and MAP above the Stage 1 hypertension level between the MgSO4 and the control groups

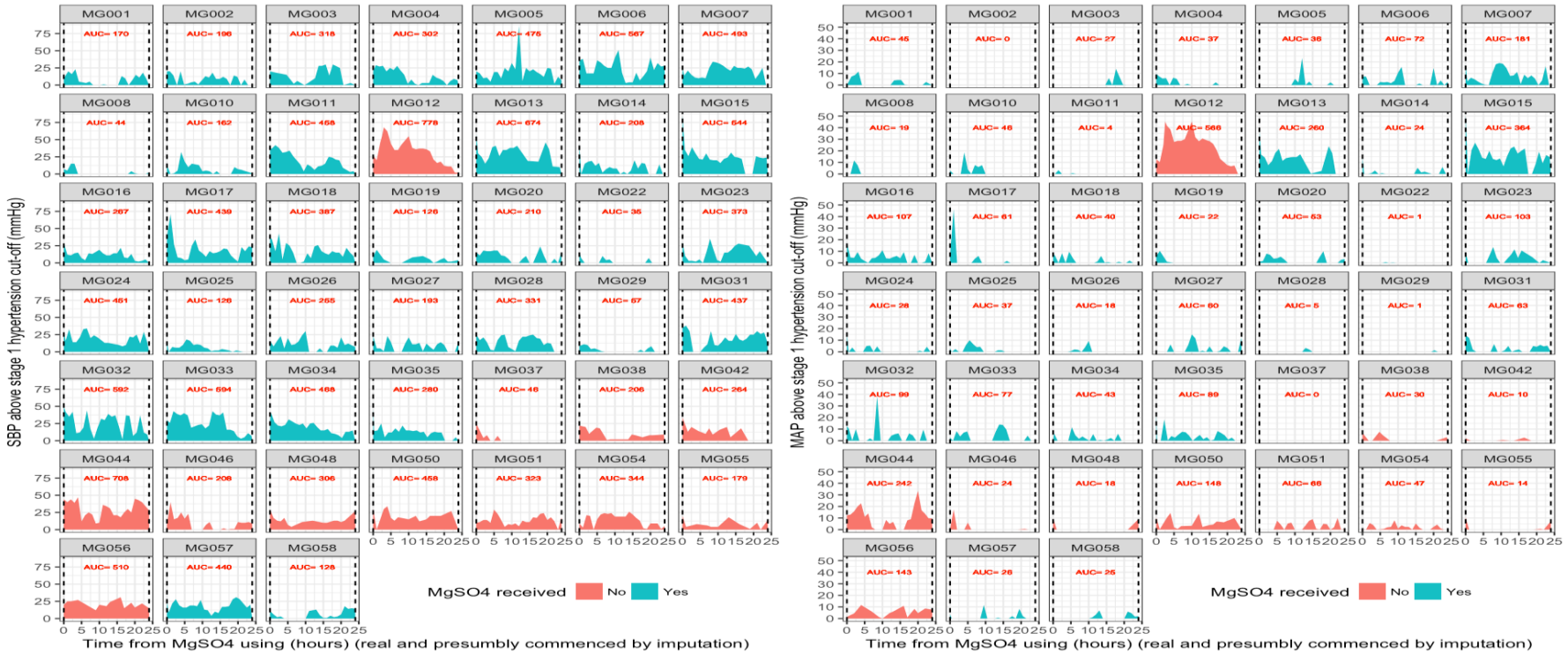
Description of AUCs for SBP or MAP in each treatment group		Estimates of difference in AUCs between treatment groups				
Groups	Median (IQR)	Parameters	Mean difference	95% CI Lower	95% CI Upper	p-value
AUC of SBP above cut-off						
		(Intercept)	140.72	16.66	264.78	0.026
Magnesium sulfate	317.8 (192.5, 458.5)	Initial SBP above cut-off *	8.27	5.29	11.25	0.000
Controls	330.5 (195.7, 509.8)	Magnesium sulfate	-46.70	-167.77	74.37	0.450
AUC of MAP above cut-off						
		(Intercept)	66.21	16.91	115.50	0.008
Magnesium sulfate	40.4 (23.5, 71.7)	Initial MAP above cut-off *	7.05	4.99	9.11	0.000
Controls	44.2 (22.8, 148.2)	Magnesium sulfate	-60.75	-113.67	-7.83	0.024

SBP: systolic blood pressure MAP: mean arterial pressure AUC: area under curve

Results were derived by pooling all 20 imputed datasets and applying Rubin's rules to calculate overall estimates.

*: SBP and MAP above cut-off at the time MgSO4 commenced

Figure 5.4: Progression of SBP (left panel) and MAP (right panel) above the relevant Stage 1 hypertension level for the first 24 hours after T=0*



*: For illustration, the presumptive times for MgSO4 administration (T=0) for the patients selected as controls are the values taken from the 12th dataset out of a total of 20 imputed datasets

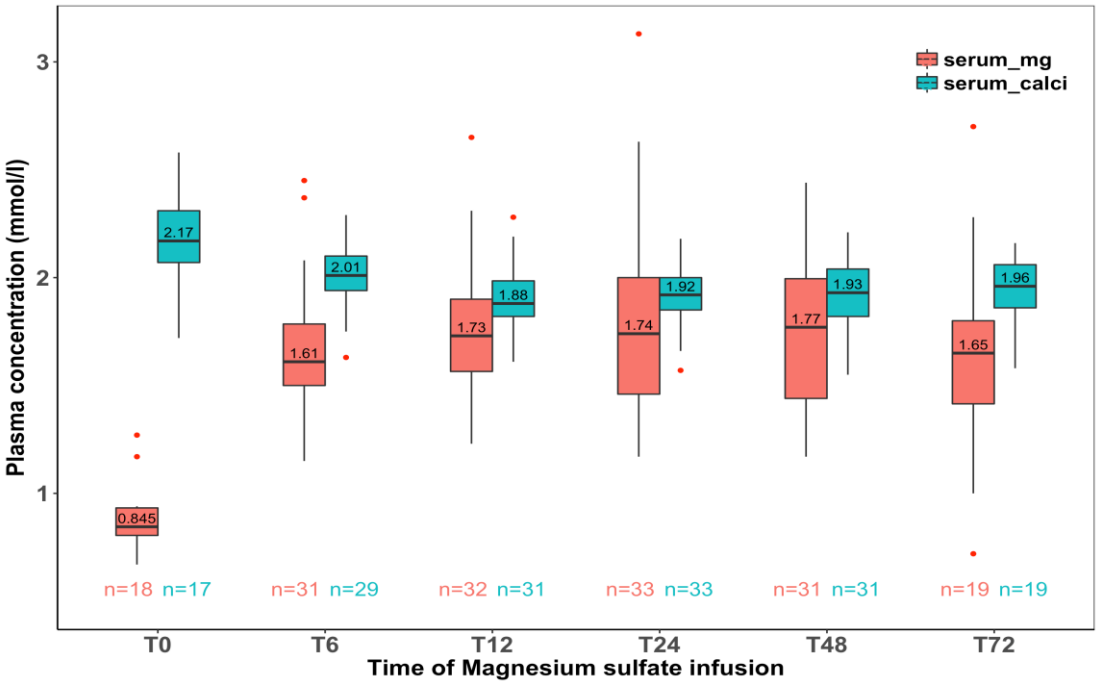
SBP: systolic blood pressure MAP: mean arterial pressure AUC: area under curve

5.3.4 Relationships between Mg/Ca levels and hemodynamic parameters

Magnesium and Calcium levels in the MgSO4 group

Baseline plasma Mg/Ca levels (taken within the 24 hours prior to commencing treatment) were measured in 18/33 (55%) of the MgSO4 group, and fell within the expected normal ranges in the majority of patients. The plasma Mg concentration increased during the first 12 hours of the infusion, and then remained fairly stable, with median values of 1.65-1.77 mmol/l for the next 48 hours. Meanwhile, plasma Ca levels decreased and fluctuated around 1.88-1.96 mmol/l with an inverse relationship to the plasma Mg levels (Figure 5.5). A single high Mg value of 3.13 mmol/l was recorded, at which there might be concern about respiratory muscle weakness. However the child had no respiratory problems and the ECG was normal.

Figure 5.5: Sequential plasma magnesium and calcium levels in the MgSO4 group.



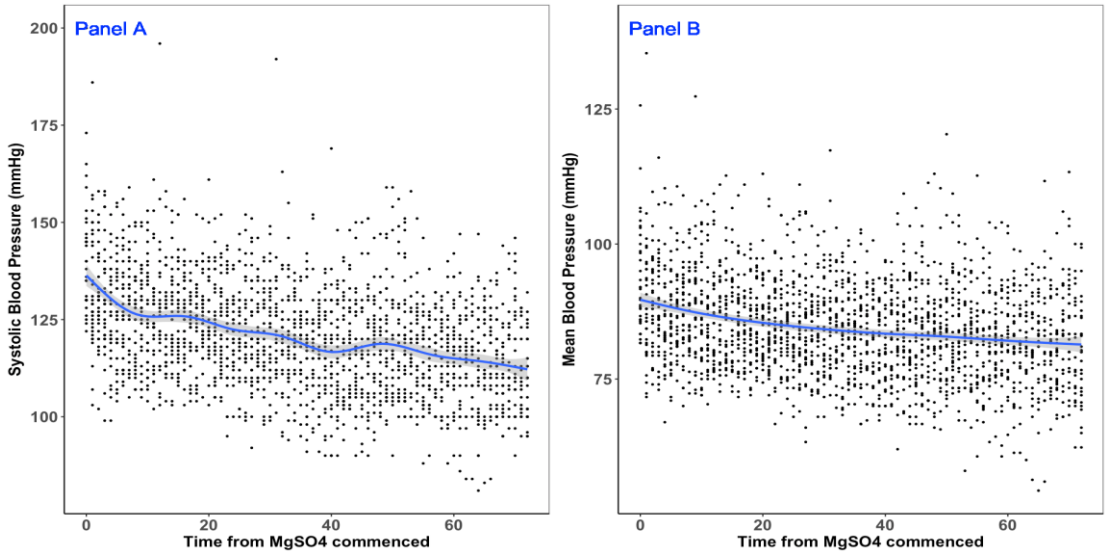
NB: Boxplots show the median and IQR values, with red dots indicating outliers

However, overall the Mg levels were lower than those measured in the Mg RCT. In this patient group values below the target concentration, i.e. less than 1.8 mmol/l, occurred in 19/32 (59%), 19/33 (58%), and 17/31 (55%) of cases at 12, 24, and 48 hours, respectively, after commencing MgSO4, while in the MgSO4 trial, the corresponding proportions were 2/13 (15%), 1/14 (7%) and 2/11 (18%), respectively. These differences likely reflect differences in the dosing regime for MgSO4 (mg/kg/hr) in the two studies, with the overall dosing schedule of the patients included in this analysis being lower that that of the RCT participants. At the mid-point of the first day of treatment, the median (IQR) dose for patients in this cohort was 40 (30, 50) mg/kg/hr compared with a dose at a similar time-point after introduction of MgSO4 of 50 mg/kg/hr in 13/14 patients in the RCT with only one child on 40 mg/kg/hr at this time. At the subsequent 24 and 48 hour assessments MgSO4 doses being given to the cohort patients were consistently slightly lower than the doses given to the RCT patients.

Relationship with hemodynamic parameters: SBP, MAP

In contrast to the increasing plasma Mg levels described above, SBP and MAP values decreased over time (Figure 5.6). The Pearson's correlation analysis shows that SBP was decreasing when MgSO4 level was increasing with the Cor (95% CI) = - 0.23 (- 0.38, - 0.07), p =0.004 (figure 5. 6). It is similar when analyzing the relation between MAP and MgSO4 levels with Cor (95% CI) = -0.27 (- 0.41, - 0.10), p= 0.002 (Figure 5.7).

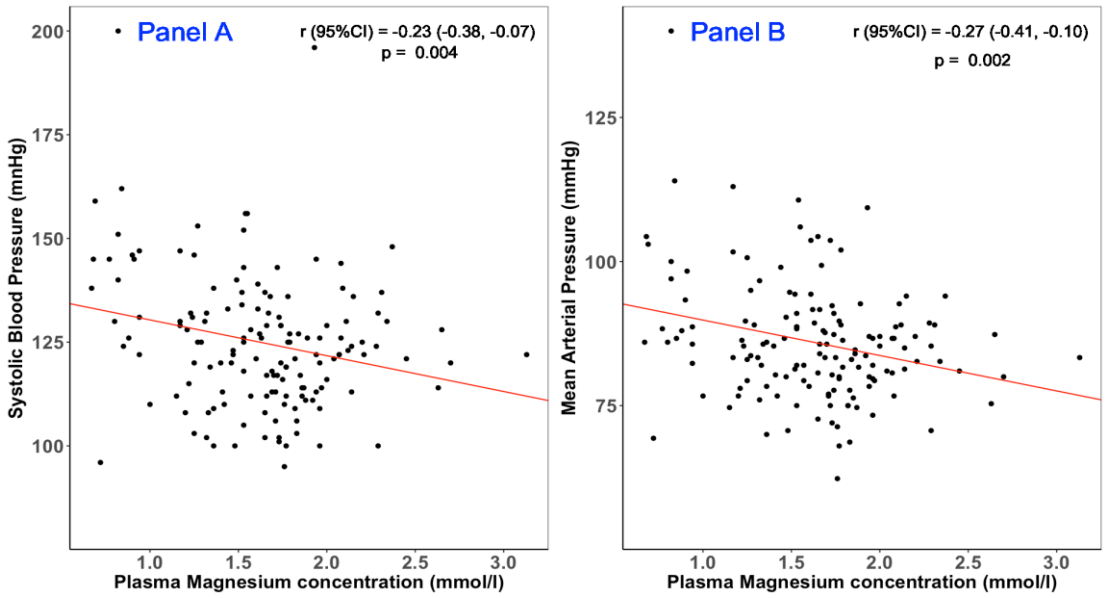
Figure 5.6: Progress of SBP (Panel A) and MAP (Panel B) over time in the MgSO4 group



Note: Individual dots represent all SBP and MAP measurements within 72 hours in the 33 cases in the MgSO4 group.

Blue lines represent the regression line of fitted values using a linear regression model (plus confidence intervals in shadowed areas)

Figure 5.7: Relationships between SBP (Panel A) and MAP (Panel B) with plasma Mg levels



Note: Individual dots represent the SBP and MAP value at each hour when Mg was measured

5.4 Discussion

Because of the small number of patients recruited into the Mg RCT, I designed this study aiming to assess the efficacy of MgSO4 using another approach. This was a retrospective analysis of all children with HFMD who achieved a certain threshold of severity. Having reviewed all the files of patients treated with high dose milrinone, I identified 33 children who had received MgSO4 as second line therapy and 12 children who had similarly severe hypertension but did not receive MgSO4, and I used these individuals as a control group. Most patients in the control group were in PICU during 2011 and the early part of 2012, while the MgSO4 group were admitted later in 2012, by which time there had been a change to the circulating enterovirus serotypes and genotypes towards less virulent viruses in general. There were some inequalities between the groups in terms of age, weight, EV-A71 status, all of which suggest that the control group might be at higher risk for a poor outcome. On the other hand however, children in the MgSO4 group were generally more severe than in control group at the time of hospital admission, and developed to the severity level for inclusion in this analysis about 1 day earlier than their counterparts in the control group; Logically, less severe disease on admission and later presentation with major complications would suggest that the overall severity might be less in the control group, including the severity of the hemodynamic instability/hypertension. However, although various of these factors (sometimes opposing each other) may have influenced the results, I could not perform a secondary logistic regression adjusting for these covariates because of the small number of patients, especially in the control group. It is also notable that none of the 6/10 (60%) cases who died in the 2011-2012 outbreak and presented with hypertension, were eligible for inclusion in this analysis because of rapid progressed to pulmonary edema and/or shock before they achieved the inclusion criteria for the study. So the findings of this retrospective analysis must be interpreted with caution.

Similar to the MgSO4 trial, we found no evidence of benefit in the group exposed to MgSO4 in terms of the primary outcome and most secondary outcomes. However, there was a significant difference in the AUCs for mean arterial pressure (MAP) above

the Stage 1 threshold, favoring the MgSO4 group, although not for systolic blood pressure (SBP). This is only one analysis out of many so may be a random occurrence, but there is a physiological rationale to support the effect observed. Magnesium ions compete with calcium ions for receptors on vascular muscle cells and can influence blood pressure by modulating vascular tone. Magnesium also has an important role in the classical pathway of NO release, with changes in extracellular magnesium content modifying production and release of NO, resulting in alterations in arterial smooth muscle tone. Magnesium also decreases the release of catecholamines after sympathetic stimulation. Consequently, there is an overall relaxation of vascular tone, with a decrease in the diastolic blood pressure (DBP) rather than SBP noticed in some studies [6, 174, 236], which would be more apparent in the MAP analysis rather than the SBP analysis. We did find weak negative correlations (but highly significant) between plasma magnesium levels and both SBP and MAP, ($r= -0.27$, $p=0.002$ and $r= -0.23$, $p=0.004$ respectively). These lines of evidence suggest that MgSO4 may have a greater impact on reducing DBP than SBP which could explain the reduction in MAP seen in the MgSO4 group compared to the controls.

It is also important to note that the patients included in this analysis were more severe than most of those enrolled in the RCT, and that the Mg concentrations measured during the drug infusions were generally lower than the levels achieved in the RCT. So a therapeutic effect may be more difficult to achieve in the patients involved in this analysis. In studies of MgSO4 use in other conditions, the target therapeutic level has varied from 2 mmol/l to 5.5 mmol/l [171, 176, 240]. In the RCT we selected a target range of 1.8 – 2.5 mmol/l, with most patients achieving the upper end of this range early on, while in the cohort of patients described here the level achieved was lower. In fact more than half the patients in this study did not achieve the 1.8 mmol/l lower margin, most likely because this was a new intervention in very sick children and the clinicians were more cautious during the first year until they developed more confidence that serious toxicity did not occur. The data from these two studies are important however in showing that potentially toxic levels of Mg very rarely result

from the infusion regime used here, although higher levels may be desired in future studies to investigate efficacy.

Given the limitations of this study as mentioned above, the findings can only be regarded as very preliminary. However, the reduction in MAP is encouraging, and supports the continued use of MgSO4 as an alternative anti-hypertensive drug in emergency situations where blood pressure can not be controlled by conventional therapy. It still remains desirable however to consider opportunities to develop further studies, especially randomized control trials, to formally investigate the efficacy and safety of MgSO4 for severe HFMD in future.

5.5 Conclusion

In summary, this study provides an overview of the experience of using MgSO4 as the rescue therapeutic for severe hypertension in patients who failed to respond to high dose conventional therapy, i.e. milrinone. Continuous infusion with a dosage of 30-50 mg/kg/hr following an loading dose of 50 mg/kg over a 20-minute infusion resulted in increased plasma Mg to within the desired therapeutic range in most cases, with the measured plasma Mg and Ca levels showing a clear dose response relationship. It was very rare to find levels above the level for potential toxicity (3 mmol/l) with this regime. Plasma Mg levels showed a weak negative correlation with SBP and MAP measurements, and the MAP assessed over 24 hours was significantly lower in the MgSO4 group than in the controls assessed as being of similar severity who did not receive MgSO4.

Even though we found no evidence of a beneficial effect in the other outcomes assessed, the patient numbers in the two groups were unequal and relatively small (especially the control group), and also there were several potential confounders at baseline so that it is not possible to say whether the two groups were really equivalent. However, the lack of any adverse effects is reassuring and supports the continued use of MgSO4 as second line therapy when managing hypertension in severe HMFD in clinical practice.

The original question of whether early intervention with MgSO4 when ANS

dysregulation first becomes apparent might control cardiovascular instability more effectively than milrinone as currently used, and thus prevent progression to severe disease, remains unanswered and a formal RCT is still needed. Alternatively, with the evidence on MgSO4 safety from these data, the alternative approach of comparing MgSO4 with milrinone directly in a formal RCT could also be considered, in order to identify the most effective regimen for children with severe HFMD who develop hypertension.

Appendix

Supplementary Table 5. 1: AUC of Systolic blood pressure above the stage 1 hypertension cut-off comparison between groups who received MgSO4 and who did not for each dataset and overall pooling

Dataset	AUC of each dataset (Median (IQR))	AUC difference after adjusting for each dataset				
				95 % CI		
		Variables	Estimate	Lower	Upper.	p
Imputation 1	317.0 (186.7, 497.0)	(Intercept)	127.885	0.177	255.594	0.050
		MgSO4 groups	-26.449	-132.594	79.696	0.617
		Initial SBP above cut-off	8.010	4.981	11.040	0.000
Imputation 2	331.7 (164.0, 498.0)	(Intercept)	122.381	1.964	242.799	0.047
		MgSO4 groups	-39.943	-144.068	64.183	0.443
		Initial SBP above cut-off	8.684	5.702	11.667	0.000
Imputation 3	302.4 (202.8, 474.0)	(Intercept)	162.211	46.598	277.824	0.007
		MgSO4 groups	-59.000	-163.449	45.449	0.261
		Initial SBP above cut-off	7.947	4.784	11.111	0.000
Imputation 4	333.0 (192.4, 482.7)	(Intercept)	141.551	26.313	256.789	0.017
		MgSO4 groups	-41.728	-141.669	58.213	0.404
		Initial SBP above cut-off	8.068	5.166	10.969	0.000
Imputation 5	336.5 (221.0, 510.0)	(Intercept)	197.288	75.426	319.150	0.002
		MgSO4 groups	-91.147	-201.843	19.549	0.104
		Initial SBP above cut-off	7.843	4.779	10.908	0.000
Imputation 6	323.2 (220.1, 498.3)	(Intercept)	148.085	21.364	274.805	0.023
		MgSO4 groups	-42.724	-146.422	60.974	0.410
		Initial SBP above cut-off	7.871	4.884	10.858	0.000
Imputation 7	333.0 (194.0, 486.7)	(Intercept)	125.541	9.392	241.691	0.035
		MgSO4 groups	-41.132	-140.882	58.618	0.410

		Initial SBP above cut-off	8.614	5.642	11.586	0.000
Imputation 8	271.4 (166.2, 475.0)	(Intercept)	96.431	-24.326	217.188	0.115
		MgSO4 groups	-7.713	-107.666	92.239	0.877
		Initial SBP above cut-off	8.462	5.344	11.579	0.000
Imputation 9	334.7 (188.2, 470.5)	(Intercept)	125.812	11.681	239.942	0.032
		MgSO4 groups	-32.023	-127.306	63.259	0.501
		Initial SBP above cut-off	8.282	5.370	11.193	0.000
Imputation 10	300.1 (217.0, 498.3)	(Intercept)	135.409	15.562	255.255	0.028
		MgSO4 groups	-49.394	-151.412	52.623	0.334
		Initial SBP above cut-off	8.558	5.609	11.506	0.000
Imputation 11	289.4 (193.1, 475.0)	(Intercept)	118.442	-1.999	238.884	0.054
		MgSO4 groups	-15.669	-116.052	84.714	0.754
		Initial SBP above cut-off	7.963	4.904	11.022	0.000
Imputation 12	314.2 (207.4, 470.9)	(Intercept)	185.142	70.973	299.311	0.002
		MgSO4 groups	-72.425	-176.780	31.930	0.169
		Initial SBP above cut-off	7.610	4.475	10.745	0.000
Imputation 13	334.7 (222.5, 498.0)	(Intercept)	166.431	48.665	284.196	0.007
		MgSO4 groups	-72.070	-174.344	30.205	0.162
		Initial SBP above cut-off	8.261	5.302	11.221	0.000
Imputation 14	280.6 (199.1, 474.6)	(Intercept)	103.081	-11.350	217.512	0.076
		MgSO4 groups	-13.945	-107.608	79.718	0.765
		Initial SBP above cut-off	8.447	5.558	11.336	0.000
Imputation 15	323.2 (192.4, 475.0)	(Intercept)	151.834	38.304	265.364	0.010
		MgSO4 groups	-61.831	-165.477	41.814	0.235
		Initial SBP above cut-off	8.416	5.237	11.595	0.000
Imputation 16	322.0 (217.1, 475.6)	(Intercept)	143.906	35.548	252.263	0.010

		MgSO4 groups	-49.855	-146.574	46.863	0.304
		Initial SBP above cut-off	8.272	5.386	11.159	0.000
Imputation 17	332.2 (198.1, 498.1)	(Intercept)	149.191	32.538	265.844	0.013
		MgSO4 groups	-56.023	-157.203	45.158	0.270
		Initial SBP above cut-off	8.304	5.426	11.181	0.000
Imputation 18	317.0 (104.0, 498.3)	(Intercept)	122.178	6.917	237.440	0.038
		MgSO4 groups	-49.374	-153.539	54.791	0.344
		Initial SBP above cut-off	9.026	6.100	11.953	0.000
Imputation 19	304.9 (207.4, 474.5)	(Intercept)	156.790	54.902	258.679	0.003
		MgSO4 groups	-69.264	-160.805	22.276	0.134
		Initial SBP above cut-off	8.504	5.728	11.280	0.000
Imputation 20	282.7 (217.1, 460.9)	(Intercept)	134.908	24.119	245.696	0.018
		MgSO4 groups	-42.264	-138.121	53.594	0.379
		Initial SBP above cut-off	8.322	5.393	11.252	0.000
Overall pooling		(Intercept)	140.72	16.66	264.78	0.026
		MgSO4 groups	-46.70	-167.77	74.37	0.450
		Initial SBP above cut-off	8.27	5.29	11.25	0.000

Supplementary Table 5. 2: AUC of Mean Blood Pressure (MAP) above the stage 1 hypertension cut-off comparison between MgSO4 group and control group for each dataset and overall pooling

Dataset	AUC of each dataset (Median (IQR))	AUC difference after adjusting for each dataset				
				95% CI		
		Variables	Estimate	Lower.	Upper	p
Imputation 1	48.6 (18.3, 147.1)	(Intercept)	58.171	10.495	105.847	0.018
		MgSO4 groups	-50.254	-100.734	0.226	0.051
		Initial SBP above cut-off	6.749	4.796	8.702	0.000
Imputation 2	50.5 (23.3, 160.3)	(Intercept)	74.111	31.118	117.104	0.001
		MgSO4 groups	-70.839	-117.717	-23.962	0.004
		Initial SBP above cut-off	7.321	5.464	9.177	0.000
Imputation 3	39.1 (21.4, 149.0)	(Intercept)	75.924	28.353	123.495	0.002
		MgSO4 groups	-68.408	-122.665	-14.150	0.015
		Initial SBP above cut-off	6.798	4.433	9.164	0.000
Imputation 4	44.2 (15.9, 149.9)	(Intercept)	52.191	7.211	97.172	0.024
		MgSO4 groups	-46.151	-94.374	2.072	0.060
		Initial SBP above cut-off	6.980	5.030	8.930	0.000
Imputation 5	55.7 (24.0, 148.2)	(Intercept)	86.829	31.445	142.213	0.003
		MgSO4 groups	-73.339	-133.072	-13.606	0.017
		Initial SBP above cut-off	6.064	3.863	8.265	0.000
Imputation 6	48.2 (25.1, 147.0)	(Intercept)	65.673	18.631	112.714	0.007
		MgSO4 groups	-58.994	-109.182	-8.806	0.022
		Initial SBP above cut-off	6.902	4.917	8.886	0.000
Imputation 7	44.2 (26.8, 145.8)	(Intercept)	73.381	33.150	113.611	0.001
		MgSO4 groups	-70.650	-115.603	-25.696	0.003
		Initial SBP above cut-off	7.387	5.544	9.230	0.000

Imputation 8	32.7 (13.4, 144.6)	(Intercept)	55.712	14.178	97.246	0.010
		MgSO4 groups	-51.809	-97.968	-5.649	0.029
		Initial SBP above cut-off	7.243	5.286	9.200	0.000
Imputation 9	40.2 (20.9, 153.5)	(Intercept)	47.652	3.422	91.882	0.035
		MgSO4 groups	-41.805	-89.230	5.620	0.082
		Initial SBP above cut-off	7.004	4.998	9.010	0.000
Imputation 10	49.6 (25.6, 147.0)	(Intercept)	76.246	33.640	118.852	0.001
		MgSO4 groups	-73.327	-120.134	-26.521	0.003
		Initial SBP above cut-off	7.364	5.503	9.225	0.000
Imputation 11	35.5 (16.7, 144.6)	(Intercept)	51.445	7.755	95.135	0.022
		MgSO4 groups	-45.353	-93.080	2.374	0.062
		Initial SBP above cut-off	6.974	4.957	8.990	0.000
Imputation 12	38.3 (17.2, 144.6)	(Intercept)	73.118	24.143	122.092	0.004
		MgSO4 groups	-63.442	-118.863	-8.021	0.026
		Initial SBP above cut-off	6.533	4.124	8.942	0.000
Imputation 13	49.9 (23.1, 160.3)	(Intercept)	78.387	34.774	122.000	0.001
		MgSO4 groups	-74.356	-122.285	-26.428	0.003
		Initial SBP above cut-off	7.227	5.317	9.137	0.000
Imputation 14	35.5 (20.5, 153.4)	(Intercept)	44.573	3.633	85.513	0.034
		MgSO4 groups	-40.758	-84.971	3.455	0.070
		Initial SBP above cut-off	7.254	5.408	9.099	0.000
Imputation 15	33.8 (22.8, 144.6)	(Intercept)	75.198	27.880	122.516	0.003
		MgSO4 groups	-67.369	-121.425	-13.312	0.016
		Initial SBP above cut-off	6.760	4.410	9.110	0.000
Imputation 16	38.3 (21.8, 151.1)	(Intercept)	60.611	21.563	99.659	0.003
		MgSO4 groups	-56.426	-100.173	-12.679	0.013
		Initial SBP above	7.208	5.405	9.011	0.000

		cut-off				
Imputation 17	49.5 (23.1, 156.9)	(Intercept)	61.656	17.042	106.270	0.008
		MgSO4 groups	-56.821	-104.268	-9.374	0.020
		Initial SBP above cut-off	7.128	5.262	8.995	0.000
Imputation 18	48.6 (23.3, 147.0)	(Intercept)	81.944	40.773	123.114	0.000
		MgSO4 groups	-79.997	-125.962	-34.032	0.001
		Initial SBP above cut-off	7.483	5.673	9.294	0.000
Imputation 19	40.2 (21.7, 151.3)	(Intercept)	62.192	21.524	102.860	0.004
		MgSO4 groups	-58.381	-103.914	-12.848	0.013
		Initial SBP above cut-off	7.254	5.318	9.190	0.000
Imputation 20	36.9 (23.9, 148.9)	(Intercept)	69.201	30.846	107.555	0.001
		MgSO4 groups	-66.569	-110.330	-22.809	0.004
		Initial SBP above cut-off	7.399	5.565	9.234	0.000
Overall pooling		(Intercept)	66.21	16.91	115.50	0.008
		MgSO4 groups	-60.75	-113.67	-7.83	0.024
		Initial SBP above cut-off	7.05	4.99	9.11	0.000

**: Overall pooling 20 dataset are calculated based on the Rubin's rule

Chapter 6

GENERAL DISCUSSION AND FUTURE RESEARCH PLANS

HFMD is still a public health issue of major concern in Vietnam and around the Asia-Pacific region. Disease incidence increased dramatically over a number of years up to 2011, and despite the recent reduction in case numbers and in clinical severity many health care practitioners remain concerned about a possible re-emergence here and/or in neighbouring countries.

Some years ago the Vietnamese MoH released guidelines for management of the disease, recommending control methods focused on hand washing as well as other ways to prevent disease transmission through the fecal/oral route. Although overall numbers have reduced from the major outbreak years of 2011-2014, mild to moderate cases continue to be seen with reasonable regularity in the outpatient departments of the big hospitals in HCMC and the possibility of a newly emerging epidemic remains real. Also, it is well recognized that the severity of disease varies over the years in part because the circulating enterovirus serotypes shift over time. If large outbreaks start to occur again, peaking over a short period, it is possible that we will again see a number of patients with rapid progression to severe disease. There is a lack of knowledge of the clinical features among these cases, which can result in an inadequate and informal evidence base with respect to management of these severe manifestations, particularly ANS dysregulation.

The first major aim of this thesis was to describe the clinical features of all severe HFMD cases seen at HTD during a defined time-period, as well as the range of outcomes and management strategies used, and to explore the predictive factors associated with severe outcomes. The second major aim was to formally assess the potential effects of intravenous MgSO4 in severe cases, focusing on hypertension control as the clearest indicator of ANS dysregulation.

Even though the clinical features of severe HFMD have been described in a few studies, most of these were based on internal classification systems [104, 190, 196, 197], or described small reviews that were mostly focused on EV-A71 [74, 125, 191, 192], or were published in local languages and therefore inaccessible to the wider scientific community [193-195]. So I focused on describing the clinical features of severe HFMD, using the data of all 1272 patients who were managed at our PICU in HCMC over 2 years. The data were gathered retrospectively, but because of the MoH guidelines that stipulate in great detail how severe HFMD cases should be monitored and managed, the data available for most cases in the series were very good, with less than 5% missing information for the parameters of interest. However there was some evolution in processes over the two-year period, so there was some variation, particularly in laboratory investigations and virological diagnostics used during the study [83, 147].

The results show that severe disease is caused not only by EV-A71, but also can be caused by CV. The predominant strains were CV-A10 and CV-A6, rather than CV-A16 as reported in southern Vietnam in 2005 and 2008, and initially in the 2011 outbreak [89, 201], and in other countries [241-243]. The shift from EV-A71 to CV infection and also in the sub-genogroups of EV-A71 from C4 to B5 [244], may help to explain the change in incidence of severe HFMD cases admitted to PICU in 2011 and 2012 compared to the overall HFMD cases in HTD. The same association between overall reduced disease severity and a shift from EV-A71 to CV species has also been shown in a recent study from China [211].

Skin lesions were more prominent in EV-A71 infection, while mouth ulcers were common in CV infection. Neurological involvement, including myoclonic jerks and focal neurological signs, were more common in cases associated with EV-A71 than CV, but this was not exclusively so. Respiratory distress occurred in one-quarter of severe cases associated with EV-A71 compared to 2% in cases related to CV. The ANS dysregulation manifestations, mostly hypertension, occurred in 16% of EV-71 cases and 2% of CV infected patients. The final stage of severe disease, i.e. pulmonary edema

and cardiopulmonary failure, occurred in 1% (PE) and 3% (cardiopulmonary failure) of the EV-A71 infections, but none of the CV-associated HFMD cases.

I also described the evolution of clinical features over time in the patient cohort. The persistence of fever for more than 3 days was associated with EV-A71 as opposed to CV infection. Other signs and symptoms such as skin lesions and mouth ulcers tended to occur concurrently with fever in the first few days of illness, similar to findings shown in previous studies [82, 83]. In contrast neurological signs rarely happened on the first day, but developed over the second and third days of illness. Subsequently manifestations of CNS involvement, particularly respiratory abnormalities, occurred from around day 3 and remained present for around 3 days. This clinical course is in keeping with the theoretical pathophysiological mechanisms that are thought to contribute to the development of severe HFMD [64].

WHO recommends that early detection and timely management are the best ways to reduce morbidity and mortality from HFMD. Some research groups have tried to investigate risk factors for progression to severe disease, aiming to identify the best strategy for follow-up and management (Table 3.1) [112, 194, 202-208]. However, in most of these studies associations with severe disease were identified, as opposed to predictive factors, because the timing of evolution of the various features was not identified. Only one study formally investigated risk prediction for neurological involvement and identified three parameters: fever persisting for 3 days; peak fever over 38 C; and a history of lethargy, as risk factors for progression [209].

As well as carefully identifying the sequence of different clinical features over time, I defined strict criteria for severe disease in my cohort and also excluded patients who deteriorated rapidly (within 24 hours of PICU admission). Using pre-defined clinical and readily available laboratory factors, I found that initial presentation with skin lesions, or with tachypnea, were predictive of subsequent deterioration to severe disease. Skin lesions are linked to EV-A71 infection, which is already an established factor causing severe disease, and was a clear predictor when included in the model. This factor was also found to be an indicator for risk of severe disease in the case-control study in Singapore [205]. Tachypnea is an early manifestation of respiratory distress, and could

also lead to severe outcome. Presentation with mouth ulcers alone was strongly associated with CV infection, and was a protective factor for severe outcome. This finding is similar to a meta-analysis of studies mainly conducted in mainland China, that showed that patients who presented with oral involvement were not at greater risk of severe disease [112], In principle, management of severe HFMD relies on early supportive care, with careful observation to recognize disease progression [83, 147]. Better detection of individuals at risk for progression should be facilitated by the results from the observational study, potentially allowing healthcare workers to direct their resources towards these high-risk groups.

Within the severe patient group, management of hypertension, a key manifestation of ANS dysregulation, is still controversial. Based on rather limited evidence milrinone has become the recommended first choice therapy for blood pressure control in Vietnam and other countries in the region [117, 147, 168]. However in 2011, when we were experiencing a very high disease burden on PICU with many severe cases, it became clear that in a proportion of these patients milrinone was not effective. Other therapeutic options were considered, mostly following conventional principles for hypertension management, including nicardipine and captopril [224], or alternatively based on anecdotal experience, i.e. hemofiltration [153, 154]. In the end, based on effective use of magnesium sulfate infusions for control of autonomic dysfunction in adults with tetanus [171], and more recently in neonates [230], I tried this therapy in a number of severe HFMD cases. Data from use of this agent as second line therapy in 19 HFMD cases with poorly controlled blood pressure despite high dose milrinone looked promising, and I therefore decided to go forward with developing a protocol for a randomized double-blind controlled trial to formally evaluate the efficacy and safety of this novel intervention for severe HFMD before it became established as the standard of care.

Finding formal evidence to support use of MgSO4 in severe HFMD is essential to fill the gaps between theoretical principles and the reality of clinical practice. However conducting a randomized blinded trial in severely ill children is a difficult undertaking. The trial was designed very carefully to ensure there were no treatment delays for the

children and that all possible safety mechanisms were incorporated in the protocol. Partly as a result of this, the trial did not actually start until mid-2014, by which time the epidemic in the region was waning and the number of severe cases was declining, probably partly reflecting a change in local enterovirus epidemiology. Case numbers continued to decline throughout 2015 and 2016, and finally only 26 participants out of a planned sample size of 190 were enrolled before the trial had to be stopped on the grounds of futility.

With the small number of participants in the trial, we found no evidence of benefit in any of the outcomes assessed, but the data were clearly not adequate to address the question of efficacy and a much larger trial is still needed to properly evaluate whether MgSO4 has a role in controlling hypertension in severe HFMD. Following on from this setback I decided to take another approach, and designed a retrospective cohort analysis aiming to make the best use of the available data on all the HFMD patients who had received MgSO4 in the preceding years, not limited to the trial participants. To do this, I identified all patients with Grade 3 HFMD and signs of ANS dysregulation with Stage 2 hypertension who had received open-label MgSO4 during the 2011-2012 outbreak, and compared their cardiovascular responses with similar cases who achieved the same basic severity level but for whom MgSO4 was not used. The numbers involved were a little larger than in the RCT, but the final results did not show any statistical difference in any major endpoints between those patients who did and did not receive MgSO4. There was a difference however, in the reduction in the AUC of mean arterial pressure, favoring those who received MgSO4. However, with the small sample size, I did not have statistical power to determine this effectively.

In both studies the safety profile of MgSO4 was carefully observed. Very little data is currently available about MgSO4 safety profiles in children, particularly in relation to measured plasma magnesium and calcium levels. There were no serious adverse events linked to magnesium usage in the retrospective cohort analysis, while the adverse events I observed in the prospective trial occurred at similar rates in both treatment groups. Plasma levels were generally consistent with the dosage titration in both studies, and in very few individuals were the plasma Mg levels outside the

desired therapeutic window. The data indicate that the dosing regimen we used, with the upper limit target of 2.5 mmol/l for plasma Mg, should be suitable for use in situations when a therapeutic effect is desired but where safety is a primary concern (e.g. in an outbreak situation, when it may not be possible to admit all patients to an ICU or DHU), and that repeated checks on plasma Mg are not necessary if there are no clinical concerns. However there may be leeway to push the upper limit target to 3 mmol/l or higher in situations where good safety monitoring is possible, for example in ventilated patients or ICU situations. Given the lack of a clear therapeutic effect, albeit in a very small study, a higher concentration should be considered, with precaution, in any future studies on HFMD.

Conducting a trial during an unpredictable outbreak is a huge challenge in terms of ethical issues, and clinical practice, for both clinical doctors and researchers [245]. As in the influenza pandemic in 2009 [246], and the Ebola outbreak in 2014 [247], testing a new therapeutic agent in an outbreak situation is often difficult. For example, without sufficient clinical information about the disease during the early phase, and without well-established study protocols together with the obvious lag time required to set up new protocols [248], many factors contribute to lost opportunities to test interventions and management strategies before the disease outbreak comes under control. We were careful to ensure the MgSO4 trial design was ethically and scientifically robust, especially in terms of safety, intending to test this novel treatment option during the massive outbreak of highly contagious HFMD, which impacted millions of young children. The trial was approved by local, national and institutional Ethical Boards, but it took time to obtain these approvals. With detailed operating procedures and good compliance from all study team members the trial was conducted well, but patient recruitment was low since disease incidence had decreased considerably by the time the trial started. Although the study termination was disappointing, the experience of developing and setting up the trial was very valuable for me and other staff, and there is now a solid foundation including the study protocol, standard operating procedures, and some preliminary data that is available for use in the future, whether in Vietnam or neighboring countries in the event of

another outbreak of HFMD. Alternatively, a modified form of this trial could be re-started quite quickly in the future.

For now, we still have a need for alternative medications that can control hypertension associated with HFMD when conventional medicines such as milrinone are not effective [147, 249]. In such circumstances $MgSO_4$ could still be considered as alternative second line therapy. Given its low price, good safety profile, and ease of availability [171, 250, 251], the question of whether $MgSO_4$ could still be optimized as first line therapy remains an important one, and if further outbreaks do occur in the region then a new trial should be considered and ideally implemented quickly.

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Appendices

Part A: Magnesium RCT Definitions, Criteria and other information

Appendix A.1: Definitions for hypertension in the study population

Hypertension in Children

The following definitions for hypertension in children are taken from the 2004 (US) national high blood pressure education program working group (NHBPEP) [147]. All percentiles refer to the relevant value for age, gender and length.

Normal BP — both systolic and diastolic BP <90th percentile

Prehypertension — systolic and/or diastolic BP ≥ 90th percentile but <95th percentile or if BP exceeds 120/80 mmHg (even if <90th percentile).

Hypertension:

- Stage 1 HTN — systolic and/or diastolic BP between the 95th percentile and 5 mmHg above the 99th percentile.
- Stage 2 HTN — systolic and/or diastolic BP > 99th percentile plus 5 mmHg.
- Hypertensive emergency: A severe symptomatic elevation in BP (> 30% compared to baseline blood pressure) WITH evidence of acute target organ damage defines a hypertensive emergency
  - Brain (seizures, increased intracranial pressure)
  - Kidneys (renal insufficiency)
  - Eyes (papilledema, retinal hemorrhages, exudates)
  - Heart (heart failure)

Hypertension in infants (6-12 months) [249]:

Because there are no normative data that describe 95th percentile BP values for infants less than one year of age, and the blood pressure is almost unchanged in infants from 6 months to 12 months, the following thresholds will be used to identify hypertension in infants:

- An invasive blood pressure measurement of >100/60 will be taken as indicating Stage 1 hypertension. (An oscillometric awake BP of > 100/60 is accepted as the

level at which follow-up BP monitoring and an evaluation for an underlying cause for elevated BP is usually recommended).

- An invasive blood pressure measurement that is persistently **≥** 110/65 will be considered as Stage 2 hypertension. (Treatment is generally initiated for BP persistently **≥** 110/65, or sooner if left ventricular hypertrophy is present)

BP Levels for Girls by Age and Height Percentile

Age	BP	SBP, mm Hg							DBP, mm Hg						
(year)	Percentile	Percentile of Height							Percentile of Height						
		5th	10th	25th	50th	75th	90th	95th	5th	10th	25th	50th	75th	90th	95th
1	50t	83	84	85	86	88	89	90	38	39	39	40	41	41	42
	90t	97	97	98	100	101	102	103	52	53	53	54	55	55	56
	95t	100	101	102	104	105	106	107	56	57	57	58	59	59	60
	99t	108	108	109	111	112	113	114	64	64	65	65	66	67	67
2	50t	85	85	87	88	89	91	91	43	44	44	45	46	46	47
	90t	98	99	100	101	103	104	105	57	58	58	59	60	61	61
	95t	102	103	104	105	107	108	109	61	62	62	63	64	65	65
	99t	109	110	111	112	114	115	116	69	69	70	70	71	72	72
3	50t	86	87	88	89	91	92	93	47	48	48	49	50	50	51
	90t	100	100	102	103	104	106	106	61	62	62	63	64	64	65
	95t	104	104	105	107	108	109	110	65	66	66	67	68	68	69
	99t	111	111	113	114	115	116	117	73	73	74	74	75	76	76
4	50t	88	88	90	91	92	94	94	50	50	51	52	52	53	54
	90t	101	102	103	104	106	107	108	64	64	65	66	67	67	68
	95t	105	106	107	108	110	111	112	68	68	69	70	71	71	72
	99t	112	113	114	115	117	118	119	76	76	76	77	78	79	79
5	50t	89	90	91	93	94	95	96	52	53	53	54	55	55	56
	90t	103	103	105	106	107	109	109	66	67	67	68	69	69	70
	95t	107	107	108	110	111	112	113	70	71	71	72	73	73	74
	99t	114	114	116	117	118	120	120	78	78	79	79	80	81	81
6	50t	91	92	93	94	96	97	98	54	54	55	56	56	57	58
	90t	104	105	106	108	109	110	111	68	68	69	70	70	71	72
	95t	108	109	110	111	113	114	115	72	72	73	74	74	75	76
	99t	115	116	117	119	120	121	122	80	80	80	81	82	83	83
7	50t	93	93	95	96	97	99	99	55	56	56	57	58	58	59
	90t	106	107	108	109	111	112	113	69	70	70	71	72	72	73
	95t	110	111	112	113	115	116	116	73	74	74	75	76	76	77
	99t	117	118	119	120	122	123	124	81	81	82	82	83	84	84
8	50t	95	95	96	98	99	100	101	57	57	57	58	59	60	60
	90t	108	109	110	111	113	114	114	71	71	71	72	73	74	74
	95t	112	112	114	115	116	118	118	75	75	75	76	77	78	78
	99t	119	120	121	122	123	125	125	82	82	83	83	84	85	86
9	50t	96	97	98	100	101	102	103	58	58	58	59	60	61	61
	90t	110	110	112	113	114	116	116	72	72	72	73	74	75	75
	95t	114	114	115	117	118	119	120	76	76	76	77	78	79	79
	99t	121	121	123	124	125	127	127	83	83	84	84	85	86	87
10	50t	98	99	100	102	103	104	105	59	59	59	60	61	62	62
	90t	112	112	114	115	116	118	118	73	73	73	74	75	76	76
	95t	116	116	117	119	120	121	122	77	77	77	78	79	80	80
	99t	123	123	125	126	127	129	129	84	84	85	86	86	87	88
11	50t	100	101	102	103	105	106	107	60	60	60	61	62	63	63
	90t	114	114	116	117	118	119	120	74	74	74	75	76	77	77
	95t	118	118	119	121	122	123	124	78	78	78	79	80	81	81
	99t	125	125	126	128	129	130	131	85	85	86	87	87	88	89
12	50t	102	103	104	105	107	108	109	61	61	61	62	63	64	64
	90t	116	116	117	119	120	121	122	75	75	75	76	77	78	78
	95t	119	120	121	123	124	125	126	79	79	79	80	81	82	82
	99t	127	127	128	130	131	132	133	86	86	87	88	88	89	90
13	50t	104	105	106	107	109	110	110	62	62	62	63	64	65	65
	90t	117	118	119	121	122	123	124	76	76	76	77	78	79	79
	95t	121	122	123	124	126	127	128	80	80	80	81	82	83	83
	99t	128	129	130	132	133	134	135	87	87	88	89	89	90	91
14	50t	106	106	107	109	110	111	112	63	63	63	64	65	66	66
	90t	119	120	121	122	124	125	125	77	77	77	78	79	80	80
	95t	123	123	125	126	127	129	129	81	81	81	82	83	84	84
	99t	130	131	132	133	135	136	136	88	88	89	90	90	91	92
15	50t	107	108	109	110	111	113	113	64	64	64	65	66	67	67
	90t	120	121	122	123	125	126	127	78	78	78	79	80	81	81
	95t	124	125	126	127	129	130	131	82	82	82	83	84	85	85
	99t	131	132	133	134	136	137	138	89	89	90	91	91	92	93
16	50t	108	108	110	111	112	114	114	64	64	65	66	66	67	68
	90t	121	122	123	124	126	127	128	78	78	79	80	81	81	82
	95t	125	126	127	128	130	131	132	82	82	83	84	85	85	86
	99t	132	133	134	135	137	138	139	90	90	90	91	92	93	93
17	50t	108	109	110	111	113	114	115	64	65	65	66	67	67	68
	90t	122	122	123	125	126	127	128	78	79	79	80	81	81	82
	95t	125	126	127	129	130	131	132	82	83	83	84	85	85	86
	99t	133	133	134	136	137	138	139	90	90	91	91	92	93	93

BP Levels for Boys by Age and Height Percentile

Age (year)	BP Percentile	SBP, mm Hg							DBP, mm Hg						
		Percentile of Height							Percentile of Height						
		5th	10th	25th	50th	75th	90th	95th	5th	10th	25th	50th	75th	90th	95th
1	50th	80	81	83	85	87	88	89	34	35	36	37	38	39	39
	90th	94	95	97	99	100	102	103	49	50	51	52	53	53	54
	95th	98	99	101	103	104	106	106	54	54	55	56	57	58	58
	99th	105	106	108	110	112	113	114	61	62	63	64	65	66	66
2	50th	84	85	87	88	90	92	92	39	40	41	42	43	44	44
	90th	97	99	100	102	104	105	106	54	55	56	57	58	58	59
	95th	101	102	104	106	108	109	110	59	59	60	61	62	63	63
	99th	109	110	111	113	115	117	117	66	67	68	69	70	71	71
3	50th	86	87	89	91	93	94	95	44	44	45	46	47	48	48
	90th	100	101	103	105	107	108	109	59	59	60	61	62	63	63
	95th	104	105	107	109	110	112	113	63	63	64	65	66	67	67
	99th	111	112	114	116	118	119	120	71	71	72	73	74	75	75
4	50th	88	89	91	93	95	96	97	47	48	49	50	51	51	52
	90th	102	103	105	107	109	110	111	62	63	64	65	66	66	67
	95th	106	107	109	111	112	114	115	66	67	68	69	70	71	71
	99th	113	114	116	118	120	121	122	74	75	76	77	78	78	79
5	50th	90	91	93	95	96	98	98	50	51	52	53	54	55	55
	90th	104	105	106	108	110	111	112	65	66	67	68	69	69	70
	95th	108	109	110	112	114	115	116	69	70	71	72	73	74	74
	99th	115	116	118	120	121	123	123	77	78	79	80	81	81	82
6	50th	91	92	94	96	98	99	100	53	53	54	55	56	57	57
	90th	105	106	108	110	111	113	113	68	68	69	70	71	72	72
	95th	109	110	112	114	115	117	117	72	72	73	74	75	76	76
	99th	116	117	119	121	123	124	125	80	80	81	82	83	84	84
7	50th	92	94	95	97	99	100	101	55	55	56	57	58	59	59
	90th	106	107	109	111	113	114	115	70	70	71	72	73	74	74
	95th	110	111	113	115	117	118	119	74	74	75	76	77	78	78
	99th	117	118	120	122	124	125	126	82	82	83	84	85	86	86
8	50th	94	95	97	99	100	102	102	56	57	58	59	60	60	61
	90th	107	109	110	112	114	115	116	71	72	72	73	74	75	76
	95th	111	112	114	116	118	119	120	75	76	77	78	79	79	80
	99th	119	120	122	123	125	127	127	83	84	85	86	87	87	88
9	50th	95	96	98	100	102	103	104	57	58	59	60	61	61	62
	90th	109	110	112	114	115	117	118	72	73	74	75	76	76	77
	95th	113	114	116	118	119	121	121	76	77	78	79	80	81	81
	99th	120	121	123	125	127	128	129	84	85	86	87	88	88	89
10	50th	97	98	100	102	103	105	106	58	59	60	61	61	62	63
	90th	111	112	114	115	117	119	119	73	73	74	75	76	77	78
	95th	115	116	117	119	121	122	123	77	78	79	80	81	81	82
	99th	122	123	125	127	128	130	130	85	86	86	88	88	89	90
11	50th	99	100	102	104	105	107	107	59	59	60	61	62	63	63
	90th	113	114	115	117	119	120	121	74	74	75	76	77	78	78
	95th	117	118	119	121	123	124	125	78	78	79	80	81	82	82
	99th	124	125	127	129	130	132	132	86	86	87	88	89	90	90
12	50th	101	102	104	106	108	109	110	59	60	61	62	63	63	64
	90th	115	116	118	120	121	123	123	74	75	75	76	77	78	79
	95th	119	120	122	123	125	127	127	78	79	80	81	82	82	83
	99th	126	127	129	131	133	134	135	86	87	88	89	90	90	91
13	50th	104	105	106	108	110	111	112	60	60	61	62	63	64	64
	90th	117	118	120	122	124	125	126	75	75	76	77	78	79	79
	95th	121	122	124	126	128	129	130	79	79	80	81	82	83	83
	99th	128	130	131	133	135	136	137	87	87	88	89	90	91	91
14	50th	106	107	109	111	113	114	115	60	61	62	63	64	65	65
	90th	120	121	123	125	126	128	128	75	76	77	78	79	79	80
	95th	124	125	127	128	130	132	132	80	80	81	82	83	84	84
	99th	131	132	134	136	138	139	140	87	88	89	90	91	92	92
15	50th	109	110	112	113	115	117	117	61	62	63	64	65	66	66
	90th	122	124	125	127	129	130	131	76	77	78	79	80	80	81
	95th	126	127	129	131	133	134	135	81	81	82	83	84	85	85
	99th	134	135	136	138	140	142	142	88	89	90	91	92	93	93
16	50th	111	112	114	116	118	119	120	63	63	64	65	66	67	67
	90th	125	126	128	130	131	133	134	78	78	79	80	81	82	82
	95th	129	130	132	134	135	137	137	82	83	83	84	85	86	87
	99th	136	137	139	141	143	144	145	90	90	91	92	93	94	94
17	50th	114	115	116	118	120	121	122	65	66	66	67	68	69	70
	90th	127	128	130	132	134	135	136	80	80	81	82	83	84	84
	95th	131	132	134	136	138	139	140	84	85	86	87	87	88	89
	99th	139	140	141	143	145	146	147	92	93	93	94	95	96	97

Appendix A.2: Inclusion and exclusion criteria

1. **Inclusion criteria**

- Age 6 months to 15 years
- Clinical suspicion of HFMD requiring PICU/HDU admission
- Considered severe enough to warrant invasive blood pressure monitoring by PICU/HDU staff
- Development of hypertension defined as follows:
  - For children aged 1 year and over, at least 3 consecutive systolic blood pressure recordings above the 95th centile for age, gender and length (USA guidelines for defining Stage 1 hypertension in children, (Appendix A.1)) measured invasively over a period of 20 minutes provided the child is not distressed or crying [252, 253].
  - For children aged 6 months to 1 year, systolic BP > 100 mm Hg measured invasively on at least 3 occasions over a period for 20 minutes provided the child is not distressed or crying
- Plus one or more of the following criteria:
  - Tachypnoea for age
  - Irregular or labored breathing, but with SpO2 above 92% in air and normal ABG (pH, pCO2, pO2, HCO_3 all within the normal range for the local laboratory)
  - Resting heart rate > 150 bpm
  - Mottled skin
  - Profuse sweating
  - Refractory fever
  - Hyperglycemia
- Informed consent

2. **Exclusion criteria**

- Past history of hypertension, chronic renal, cardiac or pulmonary disease, or any neurological disorder
- Hypertensive emergency
- Already commenced milrinone or any other inotropic agents
- Respiratory distress with SpO2<92% in air or PaCO2>45 mm Hg
- AV block or any arrhythmia
- Acute renal failure

Appendix A.3: Treatment failure criteria

1. Criteria for discontinuation of study treatment

If any of the following occur the study treatment will be stopped immediately and rescue treatment given as appropriate (Appendix B.3).

- Serious cardiac arrhythmia (eg atrio-ventricular block, prolonged QT interval)
- Hypotension: SBP < 70 + (2 X age) mmHg for 15 minutes or more
- Urine output < 1ml/kg/hr for 4 hours or more
- Cardiac arrest or any other emergency situation where the treating physician feels there is a contra-indication to the study drug.

In addition, if a patient develops respiratory distress (as defined in Appendix B.3) an urgent plasma Mg level will be performed. If intubation and ventilation is needed, or if the Mg level is > 3 mmol/L, the study treatment will be stopped and rescue treatment will be given as described in Appendix B.3.

2. Criteria for addition of milrinone

The study drug will be continued for 72 hours as described in the protocol, unless one of the criteria for discontinuation indicated above occurs. Milrinone will be commenced in the following circumstances in accordance with the VN MOH guidelines.

- Hypertensive emergency (Appendix A.1)
- SBP fails to decrease by 25% over the first 8 hours after enrolment despite maximum $MgSO_4$ maintenance infusion
- For children aged 1 year and over:
  - SBP increases to $\geq$ 99th percentile plus between 5 -15 mm Hg consistently for 30 minutes
  - SBP increases to $\geq$ 99th percentile plus 15 mm Hg consistently for 15 minutes
  - SBP increases to ≥ 40 mm Hg over baseline for 15 minutes, if this value is lower than either of the first two cutoffs. The baseline systolic blood pressure is defined as the lowest value measured at any time after admission to hospital before enrolment in the study.

- For children aged 6 months to 1 years:
  - SBP increases to ≥110 mm Hg up to to 120 mmHg consistently for 30 minutes
  - SBP increases to ≥ 120 mm Hg consistently for 15 minutes
  - SBP increases to ≥ 40 mm Hg over baseline for 15 minutes, if this value is lower than either of the first two cutoffs. The baseline systolic blood pressure is defined as the lowest value measured at any time after admission to hospital before enrolment in the study.

Occasionally the clinical status of a patient may indicate that milrinone is not the best inotrope to use – in these cases the treating physician will make management decisions appropriate to the situation.

If the patient's clinical status remains unstable after starting milrinone additional measures including ventilation and/or haemofiltration will be considered in accordance with VN MOH guidelines for management of HFMD. If the blood pressure remains high (SBP > 99th percentile plus >15 mm Hg) despite maximal doses of milrinone and study drug infusion then additional antihypertensive agents may be added – e.g. nicardipine, captopril etc. depending on the clinical scenario.

Appendix A.4: Additional study definitions

Autonomic nervous system (ANS) dysregulation: At least 2 of the following features: heart rate of 150-170 beats/min, systolic blood pressure variability with absolute values higher than the 95th percentile for age, gender and height, profuse sweating, mottled skin, respiratory abnormalities, and hyperglycemia.

For RCT we will use the following definitions for the inclusion/exclusion criteria:

Tachypnea: respiratory rate

- 6 – 12 months: ≥50
- 13 – 72 months: ≥40
- 6 – 12 years: ≥ 30
- 13 – 15 years: ≥25

Refractory Fever: Core temperature > 40^{o}C for at least 4 hours despite antipyretics

Hyperglycemia: >150 mg/dl (8.3 mmol/l) on a random test [254], or >126 mg/dl (7 mmol/l) on a fasting test (at least 4 hours after feeding).

Respiratory distress: if the patient has any one of the following findings:

- Tachypnea
- Irregular breathing
- Wheeze
- Stridor
- Cheyne-Stokes breathing
- Gasp
- Apnoeic episodes

Cardiac arrhythmia:

- Sinus tachycardia is not an exclusion criterion, but any other cardiac arrhythmia is, including AV block (Grades I-III) or QT prolonged >0.48 ms

Acute Renal failure:

- Serum creatinine > 2mg/dl (176 µmol/l) or urine output < 1ml/kg/hr for 4 hours or more

Prolongation of existing hospitalization: duration of hospitalization > 14 days

Appendix A.5: Definition of other variables:

All variables in the following tables are classified, as binary category and their value are Yes/No

Variables	Definition
Heart Rate (bpm)	For analysis: the absolute value will be used. Heart rate will be justified 10bmp per 1^{o}C above 37^{o}C
Tachycardia	For inclusion criteria: Any of heart rate > 150 bmp (after justify 10bmp per 1^{o}C above 37^{o}C
Shock	SBP < 70+ 2n and/or requirement of fluid resuscitation and/or vasopressor medicines
Irregular breathing	Irregular pattern of breathing
Stridor	Abnormal inspiration sound
Wheezing	Low respiratory rales during the expiration
Cheyne-Stoke	Irregular breathing with short apnea period (<15s)
Apnea breathing	Apnea lasting over 15 seconds
Drowsiness	GCS <15 or stupor or Legarthy manifestation
Witnessed myoclonic jerk	A jerk recorded by doctor
Cerebellar signs	Presented any of the following signs: limb tremor, nystasmus, ataxia
Paralysis	Any cranial nerves paralysis or limb paralysis that is assessed on discharge
Brainstem encephalitis	Clinical HFMD and present of myoclonus and/or ataxia and/or nystagmus and/or oculomotor palsies, and/or bulbar palsy and/or brainstems abnormality in MRI and/or abnormal respiratory pattern (irregular breathing, labor breathing and severe respiratory breathing)
Pulmonary edema	Respiratory distress and tachycardia and tachypnea and/or rales and pink frothy secretion and bilateral pulmonary infiltrates without cardiomegaly in chest radiograph
Death	Both death or moribund
Neurological sequelae at discharge	Any neurological sequels still exist at discharge
Bailey score*	Scales of Toddler and Infant Development III assessed cognitive, language and motor functions at discharge and 6 months later in cohort.

*Bayley scores derive: each aspect will be analysed and interpreted separately. The Z-scores of each aspect will be calculated by comparing the patient's score and the mean and standard deviation of the score from healthy children who matched by age, sex, maternal education level, and stunting status. Analysis using derived z-scores will also be adjusted for socioeconomic status of the mother and gender of the child.

Appendix A.6: Magnesium sulfate background information

Pharmacology:

Magnesium is important as a cofactor in many enzymatic reactions in the body. There are at least 300 enzymes that are dependent upon magnesium for normal functioning. Actions on lipoprotein lipase have been found to be important in reducing serum cholesterol. Magnesium is necessary for the maintaining of serum potassium and calcium levels due to its effect on the renal tubule. In the heart, magnesium acts as a calcium channel blocker. It also activates sodium potassium ATPase in the cell membrane to promote resting polarization and produce arrhythmias. Magnesium prevents premature labor by inhibiting myometrium contractions. In the CNS, magnesium prevents or controls seizures by blocking neuromuscular transmission and decreasing the amount of acetylcholine liberated at the end-plate by the motor nerve impulse. It also has a depressant effect on the CNS.

Safety in clinical practice

Although there are few formal research studies in young children magnesium sulphate is generally considered to be safe, even in neonates. A randomised trial that compared magnesium sulphate with placebo for women with pre-eclampsia found that exposure to MgSO4 during labor did not effect long term morbidity or mortality among the 827 children involved in the study; neonatal outcomes were similar in the MgSO4 and control patients[255]. In a Cochrane review of four studies using $MgSO_4$ for persistent pulmonary hypertension of the newborn (loading dose of 200 mg/kg MgSO4, followed by a continuous infusion of 20 to 150 mg/kg/hour lasting for 72 hours), among 40 term infants treated, no adverse events were reported except for transient bradycardia responsive to dobutamine in one of the studies. [256]. Finally, in a meta-analysis of 5 randomised controlled trials assessing use of intravenous $MgSO_4$ for treating acute asthma (182 children, doses ranging from 25 mg/kg to 75 mg/kg), the treatment was well tolerated and only minor side effects were reported, such as epigastric or facial warmth, flushing, pain and numbness at infusion site, dry mouth, and malaise [222].

Dosage:

In a randomized trial in neonates focused on management of pulmonary hypertension

the following doses were used: A loading dose of 200 mg/kg MgSO4 diluted to 10% in sterile water was given intravenously over 20 minutes, followed by a continuous infusion of 20 to 150 mg/kg/hour lasting 72 hours, aiming to obtain a concentration of serum magnesium from 3.5 to 5.5 mmol/l [257]

In a randomized trial in adults with severe tetanus: A loading dose of 2g/hour MgSO4 diluted to 10% in sterile water was given intravenously over 30 minutes, followed by a continuous infusion of 40 mg/kg/hour, lasting up to 7 days, aiming for serum magnesium levels of 2 to 4 mmol/l [171]

In severe exacerbations of asthma the following regimen is recommended by the Royal Children's Hospital in Melbourne: A loading dose of 50 mg/kg MgSO4 diluted to 10% in sterile water given intravenously over 20 minutes, followed by a continuous infusion of 30 mg/kg/hour lasting 24 -48 hours [258]

Side effects [233, 259]:

Adverse effects with magnesium therapy are primarily related to the serum magnesium level. The approximate relation between clinical manifestations and the degree of hypermagnesemia can be summarized as follows:

- Plasma Mg concentration > 1.25 mmol/l – impaired peripheral neuromuscular transmission leading to anticonvulsant effects
- Plasma Mg concentration 2 to 3 mmol/L – nausea, flushing, headache, lethargy, drowsiness, and diminished deep tendon reflexes.
- Plasma Mg concentration 3 to 5 mmol/L – somnolence, hypocalcemia, absent deep tendon reflexes, hypotension, bradycardia, and ECG changes.
- Plasma Mg concentration above 5 mmol/L – muscle paralysis, respiratory paralysis, complete heart block, and cardiac arrest. In most cases, respiratory failure precedes cardiac collapse.

Other recognised effects include:

1. Cardiovascular: Although a Mg level > 5 mmol/l may precipitate cardiac arrhythmias, magnesium is also used as a treatment for certain rhythm disturbances – such as irregular/polymorphic VT with normal baseline QT interval

2. Gastrointestinal: Abdominal cramps, diarrhea, gas formation

Dosing adjustment in renal impairment:

Patients in severe renal failure (creatinine clearance below 10ml/minute) should not receive magnesium due to toxicity as a result of accumulation. Patients with a creatinine clearance of <25 mL/minute receiving magnesium should have serum magnesium levels carefully monitored.

Appendix A.7: Suggested indications for specific interventions following Vietnamese MoH guidelines

Ventilation criteria:

- If a patient continues to display any of the following criteria despite oxygenation via nasal cannula and cardiac support with inotropic drugs for more than 60 minutes.
    - Labored breathing
    - Tachypnea with resting respiratory rate > 70 / minute without fever
    - Hypoxemia and/or fluctuating SpO2
    - Poor tissue perfusion and persistent resting HR > 180 beats/minute without fever
- Or
    - Decorticate or decerebrate rigidity
    - Coma (GCS < 10)

Hemofiltration indication criteria:

- Acute renal failure and one of the following:
    - Severe respiratory distress: Ventilation with FiO_2 > 60%, inspiratory pressure > 25 cmH_2O and PEEP > 10 cm H_2O
    - Unstable hemodynamic status despite intensive resuscitation for 3 hours
    - Coagulopathy (INR> 1,5)
    - Acute hepatic failure
    - GCS < 10

Or

- Ventilated patients with one or more of the following
    - Coma and refractory fever
    - Coma and refractory shock (shock status not improved after two hours of intensive resuscitation)
    - Heart failure or a positive Troponin I

Appendix A.8: Study schedule for all planned investigation

	Screenin	Enrolment[a] / D 1				D 2	D 3	D 4	D 5+	Discharge	Disch	M6
Hour from admission	T-x	T-1	T(-1/2)	T0	T12	T24	T48	T72	T96+			
Clinical activities												
Screening assessment												
Informed consent												
History and physical assessment												
Randomization assign and study drug												
Study drug administration												
Patient assessment									b	c	(c)	c
Recording/ downloading hemodynamic data		d	d	d/e	d/e	d/e	d/e	d/e				
Research blood test												
Chemistries		0.5 ml		1.5 ml	1 ml	2 ml	2 ml	2 ml				
Specialized additional research blood test												
Serum catecholamine				2 ml		2 ml	2ml	2 ml				
Cytokine profile				1 ml	1 ml	1 ml				1 ml		
Serology	1 ml									1 ml		
Research procedures												
ECG recording[f]												
Arterial catheterization												
Blood draw												
Diagnosis swabs												
Additional procedure												
Brain MRI									Just in stable			
Urine-Research Laboratory												
Urine Catecholamine												
Total amount of blood	1 ml	7 ml				5 ml	4 ml	4 ml		2 ml		

Note that additional clinical investigation will be performed as necessary as indication

Note: a- Enrollment stage should be done as fast as possible: 30 munite to take informed consent and check exclusion criteria, 30 minutes for drug administration.
b- Clinical assessment still be carry out daily until discharge
c- Neurological outcome and development will be assessed in this stage. Patients unable to be assessed at discharge will be invited to attend for assessment after 7 day.
d- Standard hemodynamic data will be recording/downloading and stored in PC
e- Advance hemodynamic data will be downloading from LiDCOrapid and stored in PC
f- Standard ECG recording will be performed daily and whenever an abnormality is noted on the monitor
g- indicated for 08RS stu

Appendix A.9: Definitions for laboratory abnormalities

A laboratory abnormality is one that was: 1) not present at baseline, or 2) was present at baseline and has now worsened; 3) was present at baseline, improved for >24 hours, and has now recurred, ***If the value varies during any 24 hour period, the worst grade abnormality will be recorded.***

	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
Acidosis	pH <7.37, but >=7.3	-	pH <7.3	Life-threatening consequences	Death
Alkalosis	pH >7.45, but <=7.5	-	pH >7.5	Life-threatening consequences	Death
Hemoglobin (Hgb)	6months-2yrs: 10.0 - <10.5 g/dl >2 yrs: 10.0 - < 11.5 g/dL	<10.0 - 8.0 g/dL	<8.0 g/dL	Life-threatening consequences urgent intervention indicated	Death
Platelet count decreased	<LLN - 75,000/mm3	<75,000 - 50,000/mm3	50,000 - 25,000/mm3	< 25,000/mm3	-
Cardiac Troponin I increased* Before 11Mar2015: ULN: 0.3 From 11Mar2015: ULN: 15.6 (female), 34.2 (male) pg/l	>ULN - 2.5 x ULN > 0.3 - 0.75 >15.6 – 39 (female); 34.2-85.5 (male)	>2.5 x ULN - 5 x ULN > 0.75 - 1.5 > 39– 78 (female); 85.5-171 (male)	>5 x ULN - 10 x ULN >1.5 - 3 >79– 156 (female); 171 - 342 (male)	> 10 x ULN > 3 >156 (female); 342 (male)	-
CK-MB ULN: 24 UI/l	>ULN - 2.5 x ULN (> 24- 60)	>2.5 x ULN - 5 x ULN (> 60-120)	>5 x ULN - 10 x ULN (>120 – 240)	> 10 x ULN (>240)	-
Creatinine increased ULN: <4yr : 42.2, / 4-10yr : 52.2 / >10-14 yr: 77.8 umol/l	>ULN -1.5 x ULN	>1.5 - 3.0 x ULN	>3.0 - 6.0 x ULN	>6.0 x ULN	-

Hyponatremia	<135 - 130 mmol/L	-	<130 - 120 mmol/L	<120 mmol/L	Death
Hypernatremia	>145 - 150 mmol/L	>150 - 155 mmol/L	>155 - 160 mmol/L	>160 mmol/L	Death
Hyperkalemia	>5 - 5.5 mmol/L	>5.5 - 6.0 mmol/L	>6.0 - 7.0 mmol/L	>7.0 mmol/L	Death
Hypokalemia	<3.5 - 3.0 mmol/L	-	<3.0 - 2.5 mmol/L	<2.5 mmol/L	Death
Hypermagnesemia	>1 -1.23 mmol/L	->1.23 – 2.5 mmol/L	>2.5 – 3.3 mmol/L	>3.30 mmol/L	Death
Hypocalcemia: ULN: 2.2 mmol/l (~ Ionized calcium: 1.1)	Serum calcium of <2.2 - 2 mmol/L or Ionized calcium of <1.1 - 1 mmol/l	Serum calcium of <2.0 - 1.8 mmol/L Ionized calcium of <1 - 0.9 mmol/l	Serum calcium of <1.8 - 1.5 mmol/L or Ionized calcium of <0.9 - 0.75 mmol/l	Serum calcium of <1.5 mmol/L or Ionized calcium of <0.75 mmol/l	Death
Hypoglycemia	<60 - 55 mg/dL or < 3.33 – 3.05 mmol/l	<55 - 40 mg/dL or < 3.05 – 2.2 mmol/l	<40 - 30 mg/dL or < 2.2 – 1.66 mmol/l	<30 mg/dL or < 1.66 mmol/l	Death
Hyperglycemia: ULN: 120 mg/dl or 6.7 mmol/l	Fasting glucose > 120 - 160 mg/dL or	Fasting glucose >160 - 250 mg/dL or > 8.88 – 13.88	Fasting glucose >250 - 500 mg/dL or > 13.88 – 27.8	Fasting glucose >500 mg/dL or	Death

* Note change in lab procedures on 11th Mar 2015, with new normal ranges for Troponi

Part B. Important Standard Operation Procedures (SOPs)

Appendix B.1 Screening and enrolment

PRE-SCREENING
Identify potential study patients in PICU -> • Age 6 months to 15 years • Grade 2b and 3 HFMD (clinically suspected) and medical indication for invasive blood pressure (IBP) monitoring *Open the Height Percentile Calculator on the desktop of the ward computer.* Enter the sex, age (in months) and height of the child to calculate the height percentile. Choose the appropriate *BP Label* from the study binder according to gender, age, and height percentile. **Have a different person DOUBLE CHECK all values and the chosen label.** Stick the *BP Label* on inside cover of the patient's hospital file. ❖ If the software shows the percentile such as 15th or 85th, please choose the nearest but lower percentile.
Provide Screening Number using the next available number from the *Screening and Enrolment Log.* Complete the *Screening & Enrolment Log* as the steps below are performed
Ensure that: • Arterial line/catheter is in place • Backup vein line is available beside the main line • Appropriate IBP monitoring system has been established • Standard laboratory tests (WBC, CRP, Creatinine) are being performed • Blood sugar level is recorded in the hospital observation chart every 6 (+/-2) hourly
Explain study information to the parent/guardian and give them *Informed Consent Form* (ICF) to read.
Connect the Nihon Kohden/Space Labs monitor to the LidCo rapid monitor - Refer to the *Operating Monitors SOP*. Study doctor will set up the alert at 95th percentile (from *the BP Label*) of SBP on Nihon Koden Go to menu screen, choose PRESS button. Set the alert to Emergency Signal. The monitor will alert if SBP meets the set warning levels ❖ Remember to calibrate the monitor before taking readings

If patient discharges without developing hypertension as described below, complete the *Screening & Enrolment Log* up to the column on BP.

SCREENING

If patient develops hypertension - (Grade 1 = SBP above 95th percentile, Grade 2 = SBP above 99th percentile + 5)

Verify the INCLUSION CRITERIA below with **available** observations.

- Development of hypertension defined as follows:
  - For children aged 1 year and over, at least 3 consecutive systolic blood pressure recordings above the 95th centile for age, gender and length measured invasively over a period of 20 minutes provided the child is not distressed or crying. Note the three SPB reading on the hospital file.
  - For children aged 6 months to 1 year, systolic BP > 100 mm Hg measured invasively on at least 3 occasions over a period for 20 minutes provided the child is not distressed or crying.

The thermo papers contained these readings will be printed out with signature, date of printing, and study number on them. One additional copy will be made followed the same procedure.

- Plus one or more of the following criteria
  - Tachypnea for age
  - Irregular or labored breathing, but with SpO2 above 92% in air and normal ABG
  - Resting heart rate > 150 bpm
  - Mottled skin
  - Profuse sweating
  - Refractory fever
  - Hyperglycemia (>150 mg/dl on a random test, or >126 mg/dl on a fasting test (at least 4 hours after feeding))

Verify the following EXCLUSION CRITERIA with **available** observations.

- Past history of hypertension, chronic renal, cardiac or pulmonary disease, or any neurological disorder
- Hypertensive emergency
- Already commenced milrinone or any other inotropic agents
- Respiratory distress with SpO2<92% in air or PaCO2>45 mm Hg
- AV block or any arrhythmia

• Acute renal failure: Creatinine> 2x upper normal range for age* or urine output <1ml/kg/hr for 4 hours *UNR = >6≤12 mth: 18 - 35 µ/L; >12 mth ≤12 yrs: 27 – 62 µ/L; >12 yrs: 44 – 88 µ/L
If the patient is excluded based on a permanent criteria (something that will not change) complete an SCR CRF form and the remaining information in the *Screening & Enrolment Log*. Stop all study related procedures. If the patient does not meet inclusion/exclusion criteria due to a clinical criterion that may change, continue to follow the patient and re-evaluate criteria regularly. If the patient meets all inclusion criteria and does not meet, any exclusion criteria based on available information (you may not have an ABG or ECG yet) approach the parent/guardian for informed consent.
INFORMED CONSENT - Study staff will discuss the study with the accompanying parent/guardian. If both parents are dead or not actively involved in caring for the child, the main long-term carer for the child will be accepted as a guardian and considered able to give consent for the study. Study staff will describe the purpose, procedures, risks/benefits, rights, responsibilities of participants, and alternatives to enrolment. The parent/guardian will be invited to ask questions which will be answered by study staff. If the **parent/guardian** agrees for the child to participate, they will be asked to **sign and date** an informed consent form. A copy of the form will be given to them to keep. If the parent/guardian is illiterate, the Informed Consent Form will be read to them in the presence of a witness who will sign to confirm the parent/guardian's understanding and acceptance. The **study staff** taking consent must also **sign and date** the ICF. The name of the parent/guardian and study staff (and witness if applicable) can be filled in by anyone.
REFUSAL OF CONSENT - If parents/guardians refuse consent, complete the *Screening & Enrolment Log* and an *SCR CRF*.

AFTER CONSENT IS GIVEN - If an ABG was not done within the previous 120 minutes (and showed no abnormality. Otherwise, it needs to redo), take 0.5 ml of blood. Make sure results are available as soon as possible (within 30 minutes) and check results against inclusion criteria. (Please refer to *Sampling SOP*) If an ECG was not done within the previous 120 minutes, perform a bedside ECG. Check results against exclusion criteria. In very rare cases where it is unable to perform a full ECG due to the irritability of the child and the commencing time constraint, the study doctor will observe on the monitor and check against the exclusion criteria. A full ECG is required to perform as soon as possible at later. If Creatinine was not done within the previous 4 hours (and showed no abnormality. Otherwise, it needs to redo), it needs to perform and check against the inclusion criteria.
IF THE CHILD IS NOT ELIGIBLE FOR THE STUDY - Inform the parents/guardians of the reason for exclusion. Complete the *SCR CRF* and the *Screening and Enrolment Log up to the "Enroll" column*. Place the *SCR CRF* in the *Failed Screening* section of the study binder.
ENROLMENT*
IF THE CHILD ENROLS TO THE STUDY - Print out the data stored on the ***HEMODYNAMIC PROFILE*** of each patient for the 2 hours preceding enrolment.
Take the next sequential treatment pack from the study drug storage cabinet. Write the study number from the drug package on the Screening & Enrolment Log and complete the rest of the log. Write the study number on the SCR CRF. Place the SCR CRF into a full patient file. Write the patient name and weight on the study drug package. Complete the D*elivery and Return Study Drug* form. Give treatment as soon as possible – Refer to *Medication SOP and Inpatient SOP*.
Take the T0 (Baseline) samples before the first dose of study drug if possible, including: 1.5-2ml Li-Heparin to HTD Biochemistry lab (Electrolytes, Creatinin, CKMB, Troponin, Glycemia). Clinical doctor may decide on the necessity of repeating these tests in the case of a very recently available result. 2 ml EDTA for catecholamine and 1 ml EDTA for serology and cytokine to OUCRU lab. Combined nasal/throat swab and rectal swab to. Stamp all *HTD Lab Request Forms* with the 02EI stamp. Refer to *Sampling SOP*.
Complete the *Patient Contact Sheet* in the patient file.

Do a full clinical assessment as soon as possible and complete the DEMO, HIST and EXAM CRFs.

**Make sure that the maximum time from development of hypertension to commencing study treatment is less than 30 mins for patients with Grade 2 hypertension and 60 mins for patients with Grade 1 hypertension. These times will be written on the SCR CRF (development) and the STUDRG CRF (treatment).*

Appendix B.2 Sampling

LABORATORY EQUIPMENT KIT PREPARATION

Ensure all the blood and tubes and urines aliquot are prepared in advance in a zip lock with appropriate label assigned each day. (VTM container for swabs will be stored in the fridge)

Those packages are kept in the study cabinet

Tubes	D1			D2	D3	D4	Disc
	Screening	T0	T12	T24	T48	T72	
EDTA	1	2	1	2	1	1	1
Li-Heparin	1		1	1	1	1	
Urinocol		1		1	1	1	
VTM		2					
Preservatives container 1.5 l		1		1	1	1	
Urine-Aliquot 2ml				2	2	2	

The labels for each sample are kept in the study binder.

BLOOD SAMPLING

- **To comply with the practice, all samples will be taken at 8am/8pm daily**

Ensure every time samples are taken these steps WILL be DONE:

A label with appropriate randomized number and type of specimen will be stick on the every sample tube. Note on the label with time the sample is taken, and fill in the "*Sample tracking form*"

Study nurse make sure all the necessary tests on the checklist will be ticked when performed. If not, remind the doctors immediately to give order.

During screening (ABG and Blood sugar**)**

0.5 ml arterial blood will be drawn from the consented patients for **ABG**.

Put a drop of blood from the syringe to perform **blood sugar** measurement using standard ward equipment.

Slowly push the rest to the Li-Heparin tube

Fill with "02EI screening number" on the *Label* and paste to the tube

Send it directly to Biochemical Lab with 02EI stamped on the "*Hospital indication form*" as soon as possible by study staff (nurses and medical assistants)

The Lab staff will receive sample and perform ABG as soon as possible

The result will be uploaded directly to the Hospital's network system

The study doctor will check the result in the PICU computer

In case the study staff can't receive the result after 15 minutes, she/he will make a call to Lab, mention the screening number and patient name (extension number: 290)

- If the hospital network system fails, the medical assistants will be waiting at lab to collect the result (approximately 10-15 minutes). The results will be informed to the study doctors at PICU ward (ext 226) via 02EI, or contact directly the study doctor's hand phone.

If the ABG has been performed within 120 minutes, it can be used for screening.

At T0 (enrolment)

Each enrolled patients will be test:

- Hospital Lab: 1.5-2ml Li-Heparin for Biochemistry (Electrolytes-including Mg and Ca, Creatinin, CKMB, Troponin, **Glycemia**).
- OUCRU Lab: 2 ml EDTA for catecholamine, 1 ml EDTA for serology and cytokine.

**If FBC and CRP have not been done previously, make sure these test will be performed*

Within the first 24 hours, all patients will have blood taken at 8am and 8 pm (either one may be first depending on when they are enrolled). For the next 2 days, all patients will have an 8 am draw which will be the 48 and 72 hour draws.

At T12 (if this draw comes less than 6 hour from the enrolment time, it will be delayed to the next nearest 8am/8pm draw)

Ensure each patient will have blood taken on time regard to the enrolment time (+/- 30 minutes).

- For hospital lab: 1 ml Li-Heparin for Biochemistry (Mg, Ca).
- For OUCRU: 1 ml EDTA.
- A drop of blood from the syringe will be put to perform **blood sugar** measurement using standard ward equipment. The result will be recorded in hospital file.
- A request for Ca & Mg test will be separated from other forms. On the request for Ca &Mg should include MSMD contact with a stamps “Do Not upload this result”, and a red stamp “DO NOT RETURN THIS RESULT TO PICU”

At T24

Ensure each patient will have blood taken at 8 am, including:

- For OUCRU: 2 ml EDTA for catecholamine, and 1 ml for cytokine will be taken
- For hospital routine: 1.5-2ml Li-Heparin for Biochemistry (Electrolytes-including Mg and Ca, Creatinine, CKMB, Troponin, **Glycemia**)
- A drop of blood from the syringe will be put to perform **blood sugar** measurement using standard ward equipment. The result will be recorded in hospital file.
- A request for Ca & Mg test will be separated from other forms. On the request for Ca &Mg should include MSMD contact with a stamps “Do Not upload this result”, and a red stamp “DO NOT RETURN THIS RESULT TO PICU”

At T48, T72

- For OUCRU: 2 ml EDTA for catecholamine (at T48 ONLY)
- For hospital routine: 1.5-2ml Li-Heparin for Biochemistry (Electrolytes-including Mg and Ca, Creatinine, CKMB, Troponin, **Glycemia**)
- Put a drop of blood from the syringe to perform **blood sugar** measurement using standard ward equipment
- A request for Ca & Mg test will be separated from other forms. On the request for Ca &Mg should include MSMD contact with a stamps “Do Not upload this result”, and a red stamp “DO NOT RETURN THIS RESULT TO PICU”

<u>At T96</u>

- For OUCRU: 2 ml EDTA for catecholamine

Sample handling during storage/transport/delivery

For OUCRU sample

- Fill in the OUCRU label for the sample
- Store the samples in the fridge at 4 degree Celsius
- 02EI Study nurse or PICU medical assistant will collect all samples and deliver to the Biochemistry lab no later than 8am/8pm every.
- Record the sample information into the "*Specimen Tracking Log"*.
- Biochemistry lab technician will handle the samples within 30 minutes
- Blood will be centrifuged at 3000 rpm for 5 minutes.
  Separate plasma and cell into different tubes: 1 tube for cytokine, 1 tube for serology, 1 tube for cells
- Note the date on the provided label and stick to the aliquots.
- Record the sample information into the "*Specimen Tracking Log"*.

OUCRU lab will collect the aliquots together with "Specimen Tracking Log" every day.

URINE SAMPLING

During the study

Urine will be taken at 6 am and sent to OUCRU Lab at 8 am everyday

Equipment preparation:

- Five 1.8 liters of urine collecting bottle contain 10 ml of 6M HCL (will be prepared in OUCRU Lab and send to the PICU in advance).
  - Aliquot 10 ml of 6N HCL solution and pour to five 1.5 liter bottles and store at OUCRU lab by Ms Duyen
  - Check pH every month to make sure the preservative solution is stable. If the pH changes, replace new solution in the prepared bottles.
  - Study nurse will maintain to ensure there are always five bottles available at PICU
- Make sure to shake the bottle before using.

Urine collection:

- Take one bottle and paste the label with correct time and put at bedside
- Attach the urine collecting bag to the patients (Urinocol- B/Braun)
- Make sure the study nurse will take the urine out of the collecting bag when it is nearly full.
- Empty the urine bag to the sample urinal jug with measurement.
- Measure the volume of urine at least every 4 hours and then pour the urine into the collecting bottle and shake regularly
- Record the total volume at the end of the 24 hour period on the *Nursing Chart*
- Take 2 aliquots of urine (1.5 ml), fill in the OUCRU label for the sample and "*Sample Tracking Form"* and then send to the OUCRU lab (EVI group) every day for storage at -70^{o}C.

Upon completing, study nurse will the collecting bottle and handing to the OUCRU to clean and reused.

DIAGNOSTIC SPECIMENS

After enrolment- The throat swab and a rectal swab for PCR diagnostics are taken.

The study nurse will maintain and prepare the swabs when the count is low

If the swabs:

- Are taken <u>before 4 pm</u>
  - Fill in the OUCRU label for the sample
  - 02EI Study nurse or PICU medical assistant will deliver OUCRU lab (EVI group).
  - EVI technician will handle the samples
  - Record the sample information into the "*Specimen Tracking Log"*.
- Are taken <u>after 4 pm</u>
  - Fill in the OUCRU label and date for the sample
  - Store the samples in the PICU's fridge at 4 degree
  - Record the sample information into the "*Specimen Tracking Log"*.

The study nurse will deliver the sample to virology lab at 8 am (+/-2) next day (EVI group-lab staff will be in charge) in the morning every day.

SAMPLING SCHEDULE

		D1		D2	D3	D4	D5	Disc
Hour		T0	T12	T24	T48	T72	T96	
Bichemistry (Li-Heparin)	Cre, Na, K, Cl, Ca&Mg(separated request form), TnI, CK-MB, Glucose: 1.5 ml	✓		✓	✓	✓		
	Mg/Ca only: 1ml		✓					
	ABG (heparin): 0.5ml	✓		✓	✓	✓		
OUCRU 3ml (EDTA)	2ml for catecholamine	✓		✓	✓		✓	
	1ml for cytokine	✓	✓	✓				✓
Serology		✓						✓
Urine output 5ml				✓	✓	✓		
Diagnostic specimens (nasal/throat and rectal swabs)		✓						
Amount of blood needed (ml)		5 ml	2 ml	5 ml	4 ml	2 ml	2 ml	2ml
Total (day)		7 ml		5 ml	4 ml	2 ml		2ml

Appendix B.3 Medication

Study doctor will set up the alert threshold of SBP on Nihon Koden

1. Go to menu screen, choose PRESS button, set up the upper and lower range of SBP based on the BP label that is attached in the hospital file
    - A> If the patients enrolled at hypertension stage 1 ($\geq 95^{th}$ percentile, $\leq 99^{th}$ % +5), the upper alarm will be set at 99^{th} percentile + 5 mmHg, the lower alarm will be set at 70 + 2*Age.
    - B> If the patients enrolled at hypertension stage 2 ($>99^{th}$ + 5 mmHg), the upper alarm will be set at 99^{th} percentile + 15 mmHg, the lower alarm will be set at 70 + 2*Age.
2. Set the alert to Emergency Signal. The monitor will alert if SBP meets the set warning levels

Study doctor will write order in the hospital file ("*Phiếu điều trị"*):

- Study drug: 45 ml
- 0.5 ml x weight (kg) infuse in 20 minutes
- then infuse with 0.3 ml x weight (kg)/hr continuously.

Dose will be adjusted according to clinical scenarios below and noted in hospital file as they occur.

Prepare study drug as follows:

- Mix 3 study drug vials (10 ml) and 15 ml NaCl 0.9% in a 50 ml syringe to obtain 45 ml of final preparation
- Calculate the Loading Dose according to the patient's weight

Study drug	**Volume**	**Infusion time**	**Rate (ml/hr)**
Loading dose	0,5 ml x weight (kg)	20 minutes	0,5 x weight x 3

- Set the rate of the infusion of the pump.
- Start infusion and set pump alarm to alert after 20 minutes.

(Prepare the calculation below before the 20 minute alarm alerts)

- Calculate the Maintenance Dose according to the patient's weight

Study drug	**Rate (ml/hr)**	**Infusion time**
Maintenance dose (continuous)	0,3 ml x weight (kg)	Up to 72 hours

Study drug is constantly infused following the doctor's order. The study nurse will write down in the hospital file ("*Phiếu thực hiện thuốc"*) and record any new dose on the "*Nursing chart*".

The study staff will monitor closely drug's volume and prepare the new dose in advance when the remaining volume in the syringe is around 2 ml.

- If the study bottle is broken, the study nurse will prepare the new final solution and note on the *Drug Delivery and Returning* form of number vial broke.

DOSE ADJUSTMENT

In addition to the study scheduled blood pressure monitoring, study nurses will monitor the blood pressure each hour and during acute changes according to clinical care. The study doctor will be available at the bed side to decide the dose adjustment according to the following:

❖ **During the 72 hours**

		Dose adjustment	
SBP status	**Value**	**Without MSMD advices**	**With MSMD advices***
if SBP is decreasing	> 90th and ≤95th percentiles	No Change	*Increase:* follow if not on maximum dose. *Decrease:* follow *No change:* follow
	≤ 90th percentile and ≥ 70th percentile +2n	Decrease 0.1 ml/kg for every 15 minutes	*Increase:* don't follow. *Decrease:* follow if not at the minimum dose (0.1 ml/kg) *No change:* don't follow
	rapidly more than 25% over 15 minutes	Decrease 0.1 ml/kg for every 15 minutes	*Increase:* don't follow. *Decrease:* follow *No change:* don't follow
	but fails to decrease by 25% over the first 8 hours despite maximum dose achieved	Add milrinone	*Increase:* don't follow. *Decrease:* follow *No change:* follow
If SBP is stable or unsignifically increasing	> 95th percentile and ≤ 99th centile +5 and dose is less than 0.5 ml/kg/hr)	increase 0.1 ml/kg for every 15 minutes	*Increase:* follow if not on maximum dose. *Decrease:* follow *No change:* don't follow if not on maximum dose
If SBP is increasing significantly	between 99th centile +5-15 mmHg within 30 mins	increase 0.1 ml/kg for every 15 minutes	*Increase:* follow if not on maximum dose. *Decrease:* follow *No change:* don't follow if not on maximum dose

	between 99th centile +5-15 mmHg for > 30 mins	• Continue to increase 0.1 ml/kg for every 15 minutes if dose is less than 0.5 ml/kg/hr • And add milrinone	*Increase:* follow if not on maximum dose. *Decrease:* follow *No change:* don't follow if not on maximum dose
	SBP > 99th centile +15 mmHg within 15 mins	increase 0.1 ml/kg for every 15 minutes	*Increase:* follow if not on maximum dose. *Decrease:* follow *No change:* don't follow if not on maximum dose
	SBP > 99th centile +15 mmHg for >15 mins	• Continue to increase 0.1 ml/kg for every 15 minutes if dose is less than 0.5 ml/kg/hr • And add milrinone	*Increase:* follow if not on maximum dose. *Decrease:* follow *No change:* don't follow if not on maximum dose
If SBP is increasing rapidly and there are evidences of acute target organ damage	SBP > baseline + 40 mmHg	• Continue to increase 0.1 ml/kg for every 15 minutes if dose is less than 0.5 ml/kg/hr • And add milrinone	*Increase:* follow if not on maximum dose. *Decrease:* follow *No change:* don't follow if not on maximum dose

❖ **If patients are currently on both study drug and milrinone, when need to adjust the dose, please be noticed**:

- ***Increase the dose***: Increase the dose of milrinone first until reaching the maximum dose (0,75 µg/kg/minute). If SBP still not be controlled, please inform and discuss with PI before considering unblinding.
- ***Decrease the dose***: decrease the dose of milrinone first until reaching the minimal dose (0,4 µg/kg/ minute), followed by decreasing the dose of study

drug

- **If a patient is treated with the maximum dose of milrirone as well as the maximum dose of study Mg/Placebo and the blood pressure is not controlled:** study doctors may consider to unblind the study medication to ensure the patient is receiving magnesium sulfate. Please discuss all decisions to unblind with PI, and follow the *Unblinding Procedure* below.

Please note:

Hypertensive emergency- A severe symptomatic elevation in BP (> 30% compared to baseline blood pressure) WITH evidence of acute target organ damage defines a hypertensive emergency

- Brain (seizures, increased intracranial pressure, paralysis)
- Kidneys (renal insufficiency)
- Eyes (papilledema, retinal hemorrhages, exudates)
- Heart (heart failure)

- **Dose stopping after 72 hours of study: -** *Patients who complete the 72 hour study drug treatment at a dose of:*
  - 0.3 ml/kg/hr or less may stop study drug if their clinical condition permits
  - >0.3 <0.5 ml/kg/hr should have the study drug tapered to 0.3 ml/kg/hr then stopped. Tapering should be done according to the patient's clinical condition and completed within 6 hours when possible.

**In rare cases, if study doctor is not available at the bed side when a clinical decision is needed, study nurse will contact the study doctor by phone to discuss the patient parameters. The study doctor will make a decision based on the pulse, RR, BP, Sp02 and other required clinical information. If the doctor decides to change the medication, the nurse will note this in the nursing record and the doctor will note this in the hospital file when s/he returns to the ward.*

EMERGENCY SITUATION

If the emergency situation occurs as describe as below, the study drug will be stopped immediately, the unblinding procedures must be followed and rescue management should be applied:

1. Cardiac arrest, AV block, QT >0.48ms
2. Urine output < 1ml/kg/hr > 4 hrs with evidence of acute renal failure in ultrasound and creatinine result
3. SBP < 70 mmHg

4. Intubation / ventilation

1.1 Prolonged the QT interval or any cardiac arrhythmia:

When plasma Mg level is high, usually above 5 mmol/l, this may result in prolongation of the QT interval (> 0.48 ms) and this could precipitate an arrhythmia. An ECG will be done at baseline and then daily and the QT interval will be measured as a routine. In addition if any arrhythmia is noted on the cardiac monitor the following actions will be taken.

- Assess the patient clinically, perform a full ECG, check plasma Mg and Ca level and discuss the clinical situation with the site PI.
- If the QT interval is above 480 mms, the study drug will be stopped immediately.
- Any other serious cardiac arrhythmia will be managed according to APLS guidelines
- The results of the Mg/Ca levels will go to the independent doctor for review. He/she will discuss the situation with the treating doctor. If the clinician has any clinical concerns the independent doctor will release the blood results so that the treating doctor can take any necessary action immediately. However, if the treating doctor and the site PI consider that the arrhythmia is minor (e.g occasional SVE's) and there are no other clinical issues of concern, then the blood results will not be released. The independent doctor may recommend adjustments to the study drug infusion according to the blood test results, but sham adjustments will also be made to the placebo arm to maintain blinding.
- However, if the calcium level < 0.9 mmol/l, the independent doctor will inform the treating doctor of this result specifically so that a rescue dose of 0.5ml/kg of calcium gluconate 10% can be given.

1.2 Cardiac arrest

In the event of a cardiac arrest the following actions will be taken:

- Stop study drug infusion immediately
- Emergency resuscitation will be started immediately according to APLS guidelines
- Plasma Mg and Ca levels will be checked urgently and the results will be returned directly to the ward clinicians.
- A rescue dose of 0.5ml/kg of calcium gluconate 10% will be given while awaiting the results if other attempts including CPR and conventional resuscitation drugs fail to restore sinus rhythm and effective cardiac output.
- The plasma Mg/Ca results may unblind the clinical team to the randomization arm and the fact that an urgent level has been reported to the ward doctors will be recorded in the CRF.
- Further intensive intervention will be done according to the Vietnamese MOH guidelines for HFMD management and APLS resuscitation guidelines.

2. Urine output < 1ml/kg/hr: - *define as urine output < 1ml/kg/ hr over 4 hours.*

If the urine output is < 1ml/kg/ hr over 4 hours the following actions will be taken:

- A bedside ultrasound will be performed to check whether there is any urine in the bladder
- A urethral catheter will be inserted to monitor the urine output closely, and the plasma creatinine will be checked urgently
- If the creatinine has increased to twice the baseline value, the study drug will be stopped and hemofiltration/dialysis will be performed according to the MOH guidelines regardless of the plasma Mg level.
- If the creatinine is in the normal range but the bladder is empty, then the urine output will be monitored closely for 4 hours more. If the urine output is increasing during this time the study drug will be continued, but if there is no improvement the study drug will be stopped and hemofiltration/dialysis will be performed according to the MOH guidelines regardless of the plasma Mg level.
- If the creatinine is in the normal range and there is urine in bladder, the urine output will be monitoring hourly for at least the next 24 hours

3. Hypotension: - *Development of hypotension could be the natural progression of severe disease or a side effect of MgS04, especially if the plasma Mg level is above 3 mmol/l. If hypotension occurs, defined as a drop in systolic blood pressure to below 70mmHg + 2 X the age in years lasting for 15 minutes*, the follow actions will be taken:

- Study drug will be stopped immediately
- Assess the patient clinically, check plasma Mg/Ca levels and creatinine, and discuss the clinical situation with the site PI
- Start with a fluid challenge of 5ml/kg of Lactate Ringers or NaCl 0.9% over 15 mins
- Access the central venous pressure (CVP) and titrate the rate of fluid infusion based on CVP measurements and the clinical response.
- Start inotropes or vasopressors (such as dobutamine, noradrenaline or adrenaline), and continue with interventions following the Vietnamese MOH guideline.
- The results of the Mg/Ca levels will go directly to the ward clinicians. If the calcium level is < 0.9 mmol/l, regardless of the plasma Mg level, a rescue dose of 0.5ml/kg of calcium gluconate 10% will be given.
- The plasma Mg/Ca results may unblind the clinical team to the randomization arm and the fact that an urgent level has been reported to the ward doctors will be recorded in the CRF.
- If the creatinine level is increasing and the treating doctor suspects a diagnosis of acute renal failure, there may be a risk of toxicity due to Mg accumulation. Hemofiltration will be commenced according to MOH guidelines regardless of the plasma Mg level.

4. Respiratory muscle weakness - *defined as new signs and symtomps of respiratory distress and plasma Mg level is above 3 mmol/l. This is a very rare side effect of magnesium therapy and can be suggested by elevation in Plasma Mg level and the presence of respiratory distress*

If the patient develops new or worsening signs and symptoms of respiratory distress the follow actions will be taken:

- Assess the patient clinically including deep tendon reflexes, check the plasma Mg and Ca levels and an arterial blood gas and discuss the clinical situation with the site PI.
- If the patient meets the MOH ventilation criteria as indicated above, (or the treating doctor thinks that the patient needs to be intubated for any reason) the study drug will be stopped immediately,
  - The results of the Mg/Ca levels will go directly to the ward clinicians. If the calcium level is < 0.9 mmol/l, regardless of the plasma Mg level, a rescue dose of 0.5ml/kg of calcium gluconate 10% will be given.
  - The plasma Mg/Ca results may unblind the clinical team to the randomization arm and the fact that an urgent level has been reported to the ward doctors will be recorded in the CRF.
- If the patient does not meet the MOH ventilation criteria and the treating doctor and site PI agree that immediate intervention/respiratory support is not needed the patient will be observed closely for at least 60 minutes.
  - The results of the Mg/Ca levels will go to the independent doctor for review as soon as possible. If the Mg level is above 2.5 mmol/l but below 3 mmol/l, the treating doctor will be informed to reduce the infusion dose as described in the protocol. Similar sham adjustments can also be made to the placebo arm to maintain blinding.
  - If the Mg level is 3 mmol/l or above, the independent doctor will release the result and inform the study doctor to stop the study drug infusion.
  - If the Ca level is < 0.9 mmol/l the treating doctor will be informed so that a rescue dose of 0.5ml/kg of calcium gluconate 10% can be given if appropriate.
  - If the patient subsequently meets the criteria for intubation, or the PaCO2 rises >45 mmHg, the intubation steps described above will be acted upon.
- Any further interventions will be performed according to the Vietnamese MOH guidelines for HFMD management

EMERGENCY UNBLINDING PROCEDURE

If there is any question about whether the participant should be unblinded or not, the investigator is encouraged to contact one of the Principal Investigators at:

Dr. Phan tu Qui – Tel: 0942555202 , Email: phantuqui@gmail.com

Dr. Truong Huu Khanh – Tel: -----, Email: truonghuukhanh@gmail.com

When considering unblinding due to an Adverse Event all of the following three criteria should be met in order to unblind the participant's randomization:

1. The Adverse Event must be a Serious Adverse Event as defined in the protocol and duplicated above.

2. The Serious Adverse Event must be thought to be probably or definitely related to the study drug as defined in the protocol and duplicated above.
3. The treating clinician states that knowledge of the treatment arm may change the therapy provided to the participant or improve the patient's outcome.

UNBLINDING PROCEDURE

- The contact person will verify that the participant has met the criteria for unblinding and provide the unblinding information to one of the site PIs.
- One of the PIs must confirm that a serious adverse event has occurred.
- One of the PIs will review the above criteria for emergency unblinding and confirm that knowing the treatment arm of the study will aid or change the clinical management of the participant.
- One of the PIs will complete the SAE Report Form and submit to the Data and Safety Monitoring Committee, to the manufacturers of the therapy (if the patient is randomized to the Magnesium arm of the study) and to Dr. Nguyen Van Vinh Chau, Director of HTD or Dr Nguyen Thanh Hung, Vice director of Ch1, within 24 hours of becoming aware of the event, then send reports to MOH as the above procedure.
- The PI will call the study pharmacist to request the unblinding information. The PIs will be responsible for ensuring that the unblinding is recorded in the study file by the study nurse.

Appendix B.4 Monitoring and Reporting AEs And SAEs

Definitions:

Adverse Event : An unfavourable or unexpected sign, including an abnormal laboratory finding that is temporally associated with the use of an investigational product, whether or not considered related to the product. Events will be considered Adverse Events if they occur after the first infusion of study drug commences. Pre-existing conditions that worsen after the first infusion of study drug commences will also be reported as Adverse Events.

Serious Adverse Events (SAE): In this study, an AE is a Serious Adverse Event if it results in any of the following outcomes:

- Results in death or
- Is life-threatening (i.e. the patient was at risk of death at the time of the AE) or
- Requires prolongation of existing hospitalization or
- Requires new inpatient hospitalization or
- Results in persistent or significant disability/incapacity

LABORATORY EVENTS:

All laboratory parameters will be included in the analysis, with cutoffs identifying significant abnormalities defined in the database (see section above). Significant laboratory abnormalities will be entered on an AE form only if they meet one or more of the following conditions:

- Accompanied by clinical symptoms
- Leading to a change in the study drug (dose modification or withdrawal)
- Requiring a change in concomitant therapy (e.g. dose adjustment or discontinuation)
- **Note: Laboratory abnormality only needs to be recorded as a clinical adverse event if it is associated with an intervention**

Adverse event recording

All directly observed or spontaneously reported AEs will be recorded in the participant's medical records and in the CRF and entered on the database, together with the Study Physician's assessment of the relation to the study drug. One of the PI's will be informed as

soon as possible.

Please refer to the "*AE Scale Classification*" specifically modified for 02EI to justify and record when event occur.

Adverse event reporting

- Serious Adverse Event need to be entered on the separate SAE reporting form. One of the PIs must be informed as soon as possible.
- All SAEs will be reported to the Sponsor (OUCRU), the local Research Ethics Committee (HTD or Children Hospital 1) as soon as possible.
    - **<u>For SAEs results death or threatens life</u>**
        - PI will write an initial report and send to coordinator within 2 days of the onset of event. Trial coordinator will submit the initial report to the sponsor and local IRB for comment and to office of MOH Research Ethics Committee (MOH REC) as soon as possible within 7 days.
        - The final report with comment from hospital IRB, will be sent to office of MOH Research Ethics Committee (MOH REC) within 14 days regardless the SAE has been resolved.
    - **<u>For other SAEs</u>**

PI will write an initial report and send to coordinator within 2 days of the onset of event. Trial coordinator will submit the initial report to the sponsor and local IRB.

Appendix B.4 Mg Safety Monitoring Doctor

Overview

The main responsibilities of MSMD are recording and monitoring closely the Mg/Ca value of the randomized patients and indicate if there's need to adjust the study dose following this SOP

List of MSMD:

1 –Dr Nguyen Minh Nguyet – phone number:

2 –Dr Tran Thi Van Thinh - phone number: 0907481915

3- Dr. Nguyen Thi Hoang Mai – phone number: 0989698860

A schedule of assigned MSMD (one main MSMD, and one for the substitute) will be posted in the ward. The study nurse will maintain the schedule and make arrangements with the MSDS to ensure that each MSDS is available and the responsibilities transfer smoothly. The assigned MSMD will rotate each week on Monday. Weeks should be arranged to avoid travel or leave.

Backup: If the lab/PICU cannot contact the assigned MSMD within 15 minutes of the scheduled time, they will contact the substitute with accordance to the number provided each week.

An optional tablet will be available for MSMD if needed

System set up

The *CliRes system* will be established by the OUCRU IT. It will contain the randomized allocation of each patient and randomized dose adjustments for patients who receive placebo.

Access to the *CliRes* will be strictly controlled.

ACCESS

Each assigned MSMD will be provided with a username and password to access to this CliRes database. The MSMD who is in charge each week will be responsible to entry the data on CliRes.

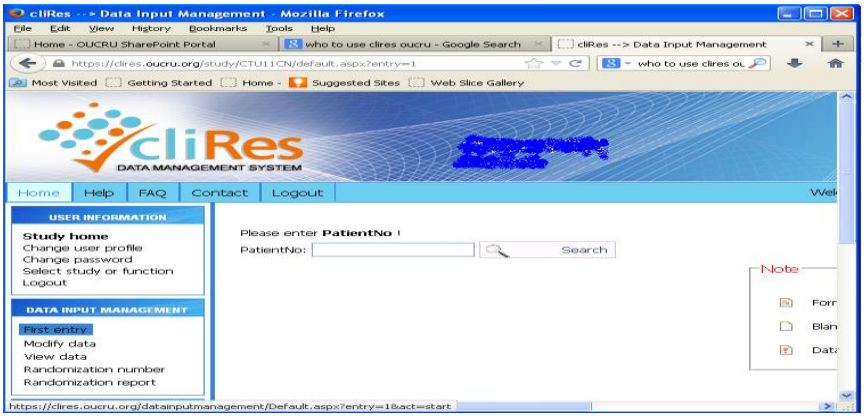
For new Patient:

Study nurse will inform MSMD about study number and patient name for any new enrolled patients

Upon receiving result of Mg/Ca, MSMD filled the click "NextPatient" to take the correct study number

For ongoing patient:

MSMD will fill in correct study number in “PatientNo” box and click “search”



MSMD will enter the value on all the required fields (i.e., date, time, Mg, Ca value, etc.), then hit the “Save” button.

CliRes can be access on any devices, and a tablet with internet access will be provided if request for a MSMD who is on duty. In case of problems with the CliRes please contact Mr. Hien, OUCRU-IT at tel: 0988084391.

ON THE WARD -

Mg/Ca levels will be checked upon enrolment, 8 am or 8 pm for the after 12 hours, and at 8 am on the next 2 sequential days.

Mg/Ca levels may be checked at any point in the case of an emergency.

Mg/Ca tests will be requested and reported separately from other Biochemistry tests. Study staff will verify the schedule to know which MSMD is on duty that day and will apply a label with the name and phone number of the assigned MSMD on the request form for Mg and Ca test. The label will also include the instruction “Do not upload this result”.

IN THE LAB – Mg/Ca tests with study labels will be processed as soon as possible. The lab staff will text and then call the number on the label to inform the result of Mg/Ca and the correlated study number to the MSMD who is in charge at that time

Lab staff who enters the lab result will make sure the Mg/Ca is not uploaded onto computer.

For the scheduled tests:

- If the Ca < 0.9 mmol/l the lab technical will inform the MSMD immediately. The MSMD will call the study/ward doctor immediately to inform them of the result.
- If the Mg>3.0 mmol/l the lab technical will inform the MSMD immediately. The MSMD will call the study/ward doctor immediately to decrease the dose.
- For any other Ca/Mg results, the lab technical will inform the MSMD no later than 10 am daily for 8 am draws and no later than 10 pm for 8 pm draws (note that this will only occur at 8 pm on days when new patients are enrolled).

MSMD

MSMD will receive message and calls from the lab according to the study schedule. The study nurse will support the MSMD to track patient schedules to know approximately when the scheduled tests/calls will occur.

When a result is received from the lab, the MSMD will repeat the result, time point and patient number back to the lab to double check the results. The MSMD will then enter the result for the appropriate patient number and time point on CliRes and enter their recommended dose adjustment (for active Mg arm only – see decision criteria below).

After receiving the schedule result, the MSMD will ring the ward and inform the guideline no later than 11 am daily or 11 pm for evening draws.

When enrolled patients are within the first 48 hours of treatment, if the MSMD does not receive a call from the lab by 10 am/pm, s/he will call the lab at 290.

MSMD will make a decision regarding the recommended dose adjustment as follows:

For patients on the placebo arm:

Call the study doctor and recommend that the dose be decreased, maintained or increased as shown on the CliRes system unless the current dose is already the maximum (when increase is shown) or minimum (when decrease is shown). In these cases, the recommendation should be No Change.

For patients on the active Mg arm:

If serum Mg <1.8 mmol/l - tell study doctor to increase dose

If serum Mg >2.5 mmol/l – tell study doctor to decrease dose.

If serum Mg 1.8 - 2.5 mmol/l – tell study doctor not to change the dose

In emergency circumstances:

- If knowing the Mg/Ca test results is relevant to affect patient care, the study/treating doctor will discuss the emergency circumstance with site PI immediately. If the study doctor/treating doctor has any clinical concerns, the result will be release immediately to the ward and the lab technician will inform the MSMD, then MSMD will complete the **Mg Safety Monitor Log**
- If not, the MSMD will follow the instruction as above

On the last recommendation (Day 4- T72): before giving the recommendation, MSMD will ask the study doctor/ clinical doctor who is on duty:

- If the study patient is still on study drug: follow the above procedure
- If the study patient completed the study drug course: no further recommendation is required. Complete the CliRes.